Studies on regeneration of watercress (Rorippa nasturtium aquaticum L. Hayek) in vitro

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STUDIES ON REGENERATION OF WATERCRESS (*RORIPPA NASTURTIUM-AQUATICUM* L. HAYEK) *IN VITRO*

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SUMMARY

This work was carried out in order to develop novel techniques for improvement of the commercial production of watercress, in the light of the problems which are presented later.

A series of experiments was carried out on the regeneration of watercress *in vitro* from various explants. Regeneration was observed only from callus, not from any organized structures. The best source of callus was leaf petioles. Shoot yields were low, even under the best conditions (12.5μM Dropp (Thidiazuron) and 0.5μM 2,4D). Maximum explant response was 31.4% and the best yield was 4 shoots per productive explant over a period of 32 weeks. The most obvious factors influencing shoot yield culture were in the light, type and concentration of PGR and choice of gelling agent. Genotype also apparently has an effect but this could not be analysed statistically.

Handling and sterilization techniques were developed for watercress flower buds. Anthers of a number of genotypes were cultured on a range of PGR (BA, 2,4D, KIN, NAA) and sucrose concentrations (5-15%) for potential production of haploids or homozygous lines. Various gelling agents and liquid media were tested and a range of thermal shock treatments was evaluated.

Three structures which may have been of microspore origin were produced from 6,378 anthers cultured. The procedure was tested and the gelling agent Gelrite was evaluated using oilseed rape as a model plant. Culture of isolated pollen on PGR-free media and of ovules on the above anther culture media was also carried out. The two calli which were formed from the 48 buds tested may have been of megaspore origin. At this stage both anther and ovule culture were limited by the lack of a plant regeneration protocol, which was subsequently developed.

Work on long term storage of shoots was carried out *in vitro* using several techniques for minimizing growth rates (low temperature, osmoticum and growth inhibitors). Storage at 10°C proved to be most effective and was a considerable improvement over the control.

Preliminary background work on the breeding system of watercress has been carried out and it has been concluded that watercress is normally self-pollinating, but in spite of this crops derived from clonal seed parents showed improvements compared to the original commercial strain. Several lines of regenerated shoots have been introduced into the commercial propagation system and grown on in a crop bed for evaluation for improved cropping characteristics.

Possible future research directions are also considered, in particular the potential for use of the protocols presented here in a disease resistance selection system.

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ABBREVIATIONS

BA	6-benzyladenine
2,4D	2,4-dichlorophenoxyacetic acid
IAA	indole acetic acid
IBA	indole butyric acid
MS	Murashige & Skoog (1962) medium
NAA	naphthaleneacetic acid
i^6Ade	6-(yy-dimethylallylamino) purine
TIBA	triiodobutyric acid
NN	Nitsch & Nitsch (1969) medium
B_5	Gamborg *et al* (1968) medium
Dropp	thidiazuron or N-phenyl-N-1,2,3-thiadiazol-5ylurea
CH	casein hydrolysate
ABA	abscisic acid
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
mRNA	messenger ribonucleic acid
SER	serine
KIN	kinetin
PGR	plant growth regulator
PP333	paclobutrazol

1. INTRODUCTION

1.1 THE BIOLOGY AND PRODUCTION OF WATERCRESS

This section presents background information about the biology of watercress and its commercial production. The limitations of the crop are discussed so that the objectives of the work described later can be seen in context.

Watercress is a plant which is usually eaten uncooked as a garnish or in salads, or cooked in soups. It is unique among the vegetables in that it commonly grows in running water (Ryder 1979). It grows both as a wild plant in streams and a cultivated crop in specially constructed beds. The first recorded cultivation of the crop in England was in 1808 by William Bradbury in Kent (MAFF 1983). It is believed to be native to the eastern Mediterranean. It was used by the Greeks and Persians, and was noted by Dioscorides as a medicinal plant in about 77 AD, and was valued mainly for its antiscorbutic properties (Howard 1976).

There are two species of watercress a diploid (2n = 32) and a tetraploid (2n = 64) and these can form a sterile triploid hybrid (2n = 48). The diploid form is *Rorippa nasturtium - aquaticum* L. Hayek formerly called *Nasturtium officinale* and the tetraploid is *R. microphylla*. The hybrid is brown cress *(R x sterilis)* which was widely grown as the crop until the 1940s.

Nowadays it is the diploid form which is grown as the crop (Howard 1976).

Watercress is a member of the *Cruciferae* and is a perennial which reproduces by both seeds and by vegetative means. Brown cress could only be propagated by vegetative means which led to a build up of virus such as cabbage black ring spot (Bleasdale 1964) and turnip mosaic virus (Tomlinson 1974). The introduction of a diploid stock of French green watercress meant that virus-free plants could be raised from seed. This strain was in use until the 1960s when a dark green watercress stock was introduced which originated from the USA. It is this stock which is still in use today (MAFF 1983).

Watercress is normally grown in chalk or limestone areas of Britain where there is an abundant supply of spring and borehole water at about 10°C. This means that production is concentrated in Hampshire, Dorset and Wiltshire. Other requirements are a stream or river as an outlet for the water from the beds and a site with a naturally high water table so that water is not lost through the base of the bed. Borehole water is important for two main reasons - one is to keep water contamination at a low level and the other is that as the water is at a constant 10°C all year round it is an important source of heat for crop growth and

protection from the frost in the winter. (Coïc *et al* 1972, MAFF 1983).

The watercress plant can be considered in three sections. It has an aerial stem portion with apex, leaves and no roots; it has a stem portion beneath the flowing water with foliage and adventitious roots growing from leaf axils; the basal part has an anchoring root system. Both root systems are capable of nutrient absorption (Cumbus 1974) but the adventitious system is dominant provided that sufficient nutrients are available in the water (Cumbus and Robinson 1977). Nutrients are translocated in adequate amounts to growing points by either system and the basal system is seen as a 'back up' system for when nutrients are low in the water (Ryder 1979). Work on cultivation of watercress in small amounts of water by Coïc *et al* (1972) indicated that watercress can grow quite adequately when supplied with sufficient nutrients e.g. in a substrate of vermiculite and watered with a nutrient solution. MAFF (1983) also state that watercress grows well in soil provided that irrigation is available and the weather is suitable.

Seed raised crops are predominantly used in spring and summer for the production of a virus-free and flower-free crop. Vegetatively propagated crops grown from stubbles or cuttings are likely to grow back in flower during these seasons which reduces their value, and

they are also prone to virus build-up. However vegetatively propagated crops are still used over the winter months as establishment of seed crops is too slow. Crop development and quality over the winter are primarily influenced by environmental rather than genetic factors so that crop improvement is better initially directed towards improvement of the seed raised crop (Rothwell (1985) pers. comm.).

Seed is produced by each grower to satisfy his own needs. At Hampshire Watercress Ltd., seed is raised over an area of 25,000m^2 from a crop planted in May. It is harvested in July, dried and cleaned yielding about 910kg of seed. This is stored in ambient temperature. It is stratified for 3-4 weeks at -20°C before sowing though there is no absolute requirement for this. A figure of £25 per 500g of seed has been suggested (Rothwell (1985) pers.comm.).

To aid germination seed is soaked for 24 hours in cold water and then for 48 hours in aerated water at 20°C. Germinated seed is mixed with an inert polyacrilamide gel, Broadleaf P_4 at a rate of about 2.2kg to 225dm^3 of polymer. Until recently this was dispensed into flat trays, but now it is placed into 20mm by 20mm cells in plastic trays. Seedlings are grown on in a glasshouse with overhead misting with a dilute nutrient solution. At about 30mm tall seedlings are transferred to a polythene covered propagation tunnel. Previous

practice involved sprinkling seedlings evenly across the gravel and at about 60mm high they would be ripped up for spreading onto crop beds. The new system leaves seedlings in small cells until they are spread onto the crop bed. This reduces mortality so that there is a saving in seed quantities and it also allows more rapid establishment on the crop bed as there is less damage to plant roots.

A new propagation system was introduced in 1988, after problems with the polymer breaking down in sunlight. Blocking compost supplied by Shamrock is dispensed into modular trays by machine. The trays contain 140 cells which are 20mm x 20mm x 40mm and are made by KIW Waalwijk (Holland). The trays are passed through an automatic washing system where they are treated with bleach between uses. Seeds are dispensed by machine at a rate of 10-20 per cell. The trays are arranged on pallets and are placed under nutrient containing mist in a glasshouse or tunnel. When the seedlings are well grown with a good root system (1-2 weeks) they are emptied into hoppers and are planted by machine. The plant clusters are placed into holders and carried on a conveyor down to the bed where they are deposited in alignment. This system minimises damage to the roots of the seedlings allowing more rapid and consistent establishment in the crop beds. However the disadvantage is that the seedlings can be prone to "damping off" soon after germination.

There are between 3,500 and 5,000 seeds per gramme (MAFF 1983, Franklin 1981). Seed germination is far from uniform and while this has been investigated by Biddington and Ling (1983) there is strong evidence for a considerable amount of heterozygosity in the crop. Calculations indicate that only 12% of the seed sown gives rise to the final crop. Stem count is between 480 and 720 stems per m^2 by harvest time. This gives some indication of the selection pressure imposed on the crop. Howard (1976) points out that repeated growing from seed is likely to involve unconscious selection for high seed production rather than for high yield of vegetative matter.

The cropping bed is constructed with concrete walls and a system of water carriers to supply borehole water at an adequate rate to the bed. A firm base to the bed is important, so it is often built on chalk. The surface layer is usually compacted gravel, as this is easy to clean and level between crops. Basic slag or a slow-release fertiliser is often added to provide minerals such as phosphorus which are not present in the water supply. Nutrients are also injected into the water supply, otherwise plants at the top end of the bed deplete the water supply and growth is limited towards the bottom of the bed.

Crop turnaround time in the summer may be as short as 3 weeks. There are two main harvest methods depending on

the market. A small machine is used to cut stems in alignment for the bunch market. This is the traditional harvest method and bunched watercress is still sold to wholesalers. Bunching watercress is very time consuming as it must be hand-cleaned and packed. It also involves damage to the plant; foreign bodies such as snails may remain trapped in the bunch; it is difficult to keep bunches in good condition as heat does not dissipate from tight bunches; and there may be a high proportion of waste. A larger machine has been designed and developed for the rapid harvesting of large areas of cress for the jumble market. Direct sales of watercress in loose prepacks are becoming a very profitable area. The plant sustains less damage, is more efficiently cleaned and the pack contains little waste. The whole process can be mostly mechanised and the crop can be kept cool more easily during packing and in transit (MAFF 1983). However crop requirements are more stringent as uneven growth results in hollows that are passed over by the cutting blade. This leads to lower yields or loose leaf being introduced into the sample. Supermarket customers have strict requirements for a consistent, high quality even crop all year round.

Crop uniformity is one of the most important factors in watercress production. This is somewhat influenced by crop nutrition, but problems with nutrient supply have largely been overcome and genotypically controlled

variation in plant vigour is the major cause of uneveness. At present a reasonably even crop is produced by introducing intense selection pressure by overplanting so that only the most vigorous plants survive to become the crop. This would seem to be selecting for those plants which grow upwards most quickly to shade out competitors, i.e. those plants with long internodes. Seed is collected from these vigorous plants in each generation. There is no selection for other characters such as leaf size, shape, colour or flowering time so there could be considerable genetic drift. Clearly the current system would have to be altered to allow any selection for characters other than vigour and rapid establishment.

Definition of the ideal plant would help in designing a method for its production and growth. It is difficult to design such a plant which must be a compromise between the product the consumer wants and the limitations of cultural techniques. Plants for the jumble market should be leafier with shorter internodes than at present. Leaf size is limited by the fact that large leaves are damaged in the mechanical harvesting process. The grower would like a quick establishing vigorous plant for rapid crop turnover in the summer, while he would like a plant that grows close to the water with vigorous tillering for the winter.

The long term commercial objective is to be able to introduce a homozygous true-breeding line into the current system which given uniform nutrition and establishment would produce an even crop. If inbreeding depression proved to be a problem F_1 hybrids could be introduced into the system. Either method would give an even crop by planting at a lower density with considerable savings on seed costs and valuable greenhouse and nursery bed space. There should be scope to be able to select for plants which more closely match the "ideal" plant i.e. short internodes, round, dark, unbroken leaves with short petioles, strong apical dominance and late flowering.

Recently a new disease problem has arisen which is causing considerable concern within the watercress industry. The disease is chlorotic leaf spot which causes unsightly yellow spots on the foliage and distortion, making the plant unsaleable. The symptoms appear with the onset of winter and disappear in May when growth of the watercress accelerates. The disease was first found in Pickering, Yorkshire in 1977 but recently has spread to Hampshire Watercress farms in Bere Regis, Dorset. The virus is carried by crook root fungus which also infects plants in the winter months. The fungus infects by means of motile zoospores and has been controlled quite effectively by addition of zinc to the water supply. However concern about levels of zinc entering the rivers from watercress beds has meant

that research into other methods of controlling crook root is vital and production of a resistant plant via a tissue culture technique such as regeneration is seen as an ideal means of control.

The development of direct sales to supermarkets has put considerable pressure on the watercress grower in that supermarkets insist on consistent high quality supplies all year round. There is also competition from an increasingly large range of alternative salad vegetables. While traditional harvest methods are labour intensive and expensive, modern mechanised techniques have meant considerable capital investment in machinery. This has led to the realisation that work must be done rapidly to overcome the limitations of the crop and regeneration from callus, or from gametes, is seen as one method of doing so.

In order to make use of techniques for regeneration it is necessary to be able to introduce regenerated plants into the commercial propagation system. Plants can then be assessed for characters such as increased disease resistance, greater yield or improved leaf morphology under normal cropping conditions.

It is also important to understand various aspects of *in vivo* growth of watercress such as its breeding system in order to allow regenerated material to be used in the most effective way e.g. as a source of

true-breeding seed, for F, hybrid production or for vegetatative propagation. Knowledge of the breeding system can also give an indication of the likelihood of regeneration from gametes, e.g. if a species is an obligate outbreeder there may be lethal recessives present which would prevent regeneration of haploids or homozygous diploids. The occurrence of inbreeding depression may prevent production of a satisfactory true-breeding line.

Howard (1976) states that most watercress seed is a result of self-pollination, while Johnson (1974) suggests that cross pollination is predominant. If self-pollination is predominant then the crop should consist of a number of lines which should tend towards homozygosity. There is clearly considerable variation within the crop which would tend to support the view that cross-pollination is predominant. It may also be that self-pollination is predominant but the considerable selection pressure imposed on watercress to produce a crop selects for the rarer but more vigorous heterozygotes.

Some preliminary experiments aimed at increasing understanding of the biology of watercress *in vivo* are described in section 3.6 and the significance of the results discussed in section 4.6.

1.2 REGENERATION IN VITRO

One of the primary goals of plant tissue culture research is crop improvement. Before cellular genetic techniques can be applied to crop improvement, efficient protocols for plant regeneration must be developed. Plant regeneration can be used to recover unique variants produced *in vitro* by techniques such as mutagenesis, protoplast fusion or cellular transformation, or naturally occurring variation can be utilized.

The totipotency of some plant cells has been widely demonstrated (Vasil & Vasil 1980) but there are considerable differences in the conditions required for regeneration, between species, genotypes and within a plant. The aim of this work was to establish the appropriate conditions for regeneration from a number of tissues of watercress i.e. callus, anthers, ovules and isolated microspores. Regeneration may either occur by direct embryogenesis or via callus formation, which must be reorganized into a shoot or embryo. Most of the *Brassica* species that have responded to anther culture undergo direct embryogenesis, but other *Cruciferae* form microspore-derived callus which must be made to undergo organogenesis. Commercial applications of regenerated plants are discussed later.

Once regeneration has been successfully carried out it may be necessary to maintain exact genotypes while they are assessed for improved cropping characteristics or other aspects such as disease resistance. Long term storage *in vitro* would be of use for this purpose and various minimal growth storage techniques are evaluated and discussed for use with regenerated plants.

The techniques for regeneration of plants are ultimately only of value if such plants can be introduced into the commercial system. For this to be achieved it is necessary to extend the current understanding of various aspects of watercress *in vivo* and some preliminary experiments have been carried out for this purpose.

1.2.1 Regeneration from Callus

Cellular differentiation in plant cells is not usually terminal, so most cells can be dedifferentiated into callus (Gautheret 1966). Wounding alone will induce cell proliferation at the surface of an intact plant which may lead to callus formation (Yeoman & Forche 1980). This usually only persists for a short time and becomes full of polyphenols to seal off the wound. Although mechanical wounding usually accompanies explantation, wounding is not necessarily a prerequisite for callus formation. This can be achieved directly with intact seedlings on the appropriate medium (Yeoman & Forche 1980).

Callus can be successfully induced from a wide range of explants, but within a given plant success may be dependent on the explant source. Seasonal variations can also affect the success of callus induction, probably as a result of seasonal fluctuation in endogenous PGR levels (Evans *et al* 1981.) or seasonal variation in inhibitory molecules (Yeoman & Forche 1980).

Response of given cells in culture is determined by environmental and genetic interactions. Differences in response and morphology may occur according to the type of explant from which the callus is derived. The size of the explant may also be important, small explants

are more likely to form callus, while large explants tend to maintain greater organogenetic potential (Evans *et al* 1981). Evans *et al* (1981) found that NAA and 2,4D are the most common auxins used in experiments for callus initiation (both used in just under half of experiments respectively). Kinetin and BA are the most common cytokinins used in callus induction (in half and a tenth of experiments respectively). 2,4D can be used alone for callus initiation but it is used more commonly in combination with a cytokinin. In general a high auxin level and low cytokinin level are used for callus initiation (Evans *et al* 1981).

Once cells have reverted to the primary meristematic state, in callus (Gautheret 1966), they can be reorganized to give a meristem and then an organ. The underlying factors involved in organogenesis are not clear because the stimuli may include components in the media, endogenous compounds produced during culture and substances carried over from the original explant (Dodds & Roberts 1985). With prolonged culture several changes occur which can include hormone habituation and loss of organogenetic potential (Murashige 1974). Correlations have been found between degree of polyploidy and capacity for organ formation (Murashige 1974).

The first indication that *in vitro* organogenesis could be chemically regulated was by Skoog (1944) who showed

that auxin could stimulate root formation in tissue cultures. Root formation is the most common type of organogenesis seen in tissue cultures. Roots can form from explants from any part of the donor plant, they are usually distributed irregularly over the callus surface and resemble the structure of intact seedling roots (Reinert *et al* 1977). Observations support the hypothesis that localised meristematic activity precedes organized development of roots and shoots (Ross *et al* 1973). Shoot buds are also formed quite frequently but usually on separate cultures to roots. Leaves are formed occasionally (Reinert *et al* 1977).

Despite numerous studies on organogenesis it is difficult to make extensive generalisations . The initiation of organs occurs most frequently on recently isolated tissue though there are exceptions (Reinert *et al* 1977). Shoot formation is achieved by transfer of callus to medium with PGRs present in different concentrations. Hormones most commonly used are NAA (a third of experiments), 2,4D (a tenth of experiments), kinetin (half of experiments) and BA (a quarter of cases). (Evans *et al* 1981) The second medium may contain an auxin with lower biological activity, cytokinin only or PGRs may be completely eliminated. 2,4D must usually be removed from the medium prior to shoot development. Commitment for organogenesis occurs in the primary medium (Evans *et al* 1981) but it is the endogenous auxin:cytokinin balance which is the key

factor (Thorpe 1980) so exogenous hormones do not always act as predicted. In general a high auxin:low cytokinin ratio is necessary for callus formation and a low auxin:high cytokinin ratio for shoot formation (Skoog & Miller 1957). A variety of related compounds can substitute for cytokinin including substituted purines, pyrimidines and ureas (Thorpe 1980). There is also limited evidence for the influence of nitrogen, light levels and sugar levels in organogenesis (Reinert *et al* 1977) though no generalisations can be made.

There is one report of the control of morphogenesis *in vitro* in watercress (Ballade 1972) which indicated that it is possible to manipulate the development of axillary meristems which were excised and placed onto medium containing various PGRs. Excision of the tissue caused a temporary cessation of organ development. Application of kinetin caused bud formation, while application of IAA caused formation of roots. There was no callus formation so this work does not constitute "de novo" organogenesis.

In the *Cruciferae* regeneration has been achieved in several species. Clare & Collin (1974) working on *Brassica oleracea* var. *gemmifera* (Brussels sprout) found low 2,4D ($0.2 mgdm^{-3}$) and kinetin ($0.5 mgdm^{-3}$) stimulated greatest callus growth while shoot bud formation was induced from sprout stem and petiole sections on $2 mgdm^{-3}$ IAA and $0.5 mgdm^{-3}$ kinetin. There

response varying with season and physiological status of the donor plants.

In *B. oleracea* var. *capitata* (Bajaj & Nietsch 1975) plants were regenerated from roots, stems, cotyledons, leaves and callus cultures. Shoot bud formation occurred on PGR-free MS medium but the frequency was increased by addition of 0.5mgdm^{-3} kinetin to the medium. Callus underwent organogenesis on 2mgdm^{-3} kinetin and 2mgdm^{-3} IAA. Pareek and Chandra (1978) induced callus in *B. oleracea* var. *botrytis* with IAA at 1mgdm^{-3} and kinetin at 0.5mgdm^{-3}. After four weeks this began to undergo somatic embryogenesis rather than organogenesis. Embryos are not regarded as organs as they have no vascular connections to the parent tissue (Vasil & Vasil 1980). Johnson and Mitchell (1978) also working on *B. oleracea* (var *italica*) produced shoots from callus originating from leaf and stem explants. They concluded that with attention to the best auxin:cytokinin ratios plants ought to be able to be raised successfully from all cultivars of *B. oleracea*.

Other reports on regeneration in the *Cruciferae* include *Arabidopsis thaliana* (Feldmann & Marks 1986), *Brassica oleracea* (Lustinec & Horak 1970, Clare & Collin 1974), *B. alboglabra* (Wong et al 1987), *Raphanus sativus* (Paek *et al* 1987). There have also been comparisons between species and cultivars of *Brassica* by Dunwell 1981, Dietert *et al* 1982, Narasimhulu & Chopra 1988.

In the majority of these reports juvenile tissue such as cotyledons and hypocotyls have been used as explants. However in watercress the small size of the seeds at 5000 per gramme (MAFF 1983) and hence seedlings meant that use of such tissues was impractical. A number of workers have induced regeneration from mature tissues such as stem, leaf and rib of broccoli (Johnson & Mitchell 1978), and leaf discs of *B. oleracea*, *B. napus* and *B. campestris* (Dunwell 1981).

There is evidence that in some species the nature of the cytokinin is an important influence on the behaviour of explants in culture. In *Acer x freemanii* (Kerns & Meyer 1986) proliferation in culture will not occur with cytokinins BA, kinetin, 2iP or auxins IBA, TIBA or combinations of these. However treatment with thidiazuron (Dropp) at concentrations of 0.05 and 0.01μM causes shoot proliferation. This compound is a substituted urea which has been reported to have cytokinin-like activity in *Phaseolus* tissues (Mok *et al* 1982) and it is suggested that thidiazuron may be of interest in other species. Mok *et al* (1982) found that thidiazuron was more effective on the growth of callus cultures of *P. lunatus* than the most active of the adenine-type cytokinins such as zeatin. The class of urea cytokinins have very similar biological activities in terms of cell division, shoot formation and inhibition of root growth to the adenine cytokinins and

results given by Kurosaki *et al* (1981) indicate that the two groups of compounds probably have a common active site.

The materials and methods employed and the results obtained are described in section 3.1, and their significance is discussed in section 4.1.

1.2.2 Regeneration from Anthers, Ovules and Pollen

The aim of work on regeneration from anthers, pollen and ovules is to produce plants from the haploid gamete. In the case of anthers and pollen this is the microspore and in the case of ovules this is the megaspore.

Haploid plants are those possessing the gametic rather than the somatic chromosome number. They may occur spontaneously in nature - first reported in 1922 by Blakeslee *et al* while working with *Datura stramonium* - or they may be induced experimentally.

The interest in haploids is mainly due to their great potential in plant breeding. Haploids have only one set of alleles at a locus so that experiments using such plants are unencumbered by problems of dominance and segregation (Maheshwari *et al* 1982). Since most mutations are recessive they can be difficult to detect in the prsence of an unmutated dominant allele, whereas

in haploids recessive mutants can be detected (Bajaj 1983). Both desirable and undesirable recessive traits are expressed in haploids and because of this a major application of haploid production relates to the greatly increased effectiveness in selection of genetic recombinants.

Doubling of the chromosome number of haploids offers a method for the rapid production of homozygous plants as an alternative to repeated cycles of inbreeding in self pollinating crops (Dunwell 1985b) and is of particular value in cross-pollinating species and dioecious species where producing inbred lines is very difficult or impossible. Homozygous lines can be produced within 6 months compared to 4-6 years, which inbreeding methods might take (Dunwell (1987) pers. comm.).

Spontaneous methods of haploid production include apomixis or parthenogenesis, while artificial methods may be by culture of anthers, pollen, ovules, protoplasts or by chromosome elimination (Bajaj 1983).

Lack of an efficient and simple technique for haploid production has proved to be a major hurdle in many species. However in over 200 species (Dunwell 1985a) anther culture techniques have proved to be relatively quick, simple and efficient (Bajaj 1983). The technique was first discovered accidentally in *Datura innoxia* by Guha & Maheshwari (1964) in experiments to

study the onset of meiosis in anthers cultured *in vitro*. This was particularly remarkable in that there was direct organized growth of embryos and this led to formation of haploid plants, each of which was derived from a single pollen grain (Guha & Maheshwari 1966).

Under appropriate conditions the microspores within, or sometimes isolated from, the anthers of responsive species can be induced to follow a pathway of sporophytic rather than gametophytic development. Sporophytic development may be by direct embryogenesis or via callus production, from which plants can be regenerated (Keller *et al* 1975).

It is important to consider the structure of the angiosperm stamen in relation to anther and pollen development. A typical angiosperm stamen consists of filament, connective tissue and anther. The anther has two lobes each of which have two microsporangia or pollen sacs. Within the anther are four patches of primary sporogeneous tissue which contain some cells acting as pollen mother cells (PMC) (Bell & Woodcock 1983). These PMCs form pollen tetrads by meiosis giving four microspores which are released (Bajaj 1983). These are densely cytoplasmic but enlarge rapidly mainly by vacuolation, and each becomes invested with a tough outer coat of sporopollenin - the exine (Sunderland & Dunwell 1977). The nucleus is pushed to the periphery. At the first mitosis the

microspore nucleus divides to produce a large vegetative and small generative nucleus. The wall separating these cells eventually becomes detached from the intine giving the unique cell-within-cell arrangement in the male gametophyte. It is after this detachment that the generative cell divides to give the male gametes in the species shedding pollen in a tricellular state eg *Cruciferae, Graminae.* In the species with bicellular pollen this division occurs in the pollen tube (Sunderland & Dunwell 1977) Work on *Nicotiana tabacum* (Sunderland & Wicks 1969) indicates that it is usually the vegetative nucleus which divides to form the embryoid tissue, though in *Hyoscyamus niger* Raghaven (1978) found that it is the generative nucleus which divides.

The key to the rapid development of anther culture was undoubtedly the recognition that angiosperm pollens can be switched from their normal developmental pathway only if cultured within a certain period of development (Sunderland & Dunwell 1977). This is fixed within a variety but may vary between cultivars and species (Nitsch 1983). This critical period, however, is between the formation of spore tetrads and the deposition of starch early in the gametophytic stage. This period has been divided into 6 stages (Sunderland & Dunwell 1977).

1. spore tetrads
2. vacuolate spores
3. spores in process of DNA replication
4. spores undergoing first pollen division
5. young pollen grains, vacuole and curved cell wall still present
6. young pollen grains, no vacuole, vegetative cells filled with cytoplasm, generative cell detached from intine. Little starch deposition.

Very responsive species will respond at all stages to some extent while others will only respond at one stage e.g. at stage one-*Arabidopsis thaliana* (Gresshoff & Doy 1972a). Exact determination of pollen stage requires cytological analysis but for large scale programmes many authors have used a simple, measured, external, morphological character such as corolla length (Keller *et al* 1983). This is also used in cases where the exine forms very early preventing further staging e.g. *Brassica napus* (Dunwell *et al* 1983) as there is no satisfactory method of removing the very inert exine.

The genotype of donor plants seems to be the major determinant of response of anthers in culture, not only species within a genus, but even cultivars within a species show markedly different responses in culture. There are two basic components of response i.e. number of productive anthers and number of regenerants per productive anther. Studies by Dunwell (1985c) on two

strains of barley and their F_1 hybrid confirmed that these two estimates of success are independently determined and under separate genetic control, though it has not been possible to link this to any known genetic locus.

Gresshoff & Doy (1972 a, b) working on 18 lines of *Arabidopsis thaliana* and 43 lines of *Lycopersicon esculentum* could only induce haploids in 3 cases each. In a recent study on 13 cultivars of maize (Bretell *et al* 1981), plants were produced from only 3 cultivars and the total yield was only 14 plants from 21,638 anthers. Bajaj (1983) suggests that one common reason for failure is that workers restrict themselves to only one or a few cultivars and points out that a general survey of cultivars should be undertaken in a new species. Dunwell (1985c) feels that the constraints imposed by choice of genotype may drastically limit the applications of anther culture. Successful regeneration of plants via anther culture has been shown in a number of members of the *Cruciferae*. These include:

Arabidopsis thaliana	Gresshoff & Doy 1972a
Brassica campestris	Keller *et al* 1975
B. chinenis	Zheng *et al* 1978
B. hirta	Leelavathi *et al* 1984
B. juncea	Sharma & Bhojwani 1985
B. napus	Keller & Armstrong 1977

B. oleracea	Keller & Armstrong 1981
B. oleracea x alboglabra	Kameya & Hinata 1970
B. pekinensis	Deng *et al* 1982
Iberis amara (callus only)	Babbar *et al* 1980

There are certain features that anther culture techniques for all these species have in common. These can be used as indications for the starting point for anther culture of other members of the *Cruciferae*.

Most of the *Brassica* species undergo direct embryogenesis which has been seen as desirable as it is quicker and less subject to the genotypic instability of callus (Keller *et al* 1975). However variation does occur in embryo-derived plants (Ockendon 1984) including polyploidy, aneuploidy etc. If the origin is a single haploid pollen grain, regenerated plants ought to be homozygous, though levels of ploidy greater than diploid would be of little practical use. Many of the embryos do not develop normally and may produce secondary embryoids on their surfaces (Loh & Ingram 1982). These could be regenerated into plants on cytokinin-containing medium (Loh *et al* 1983). Some of the *Cruciferae* eg *A. thaliana, B.hirta, I.amara* respond to anther culture by callus production from microspores.

Assuming that a responsive genotype has been found, it is believed that the growth conditions of the donor

plant can have a profound effect on the yield of microspore derivatives. Thurling & Chay (1984) found different responses with cultivars of *B. napus* grown in different temperature and light regimes, though the mechanism by which environment influences embryogenetic potential is not clear. Similar effects have been found in *B. campestris* (Keller *et al* 1983). The age of the plant has also been found to be of importance e.g. Thurling & Chay (1984) found first flowers produced more embryos than flowers which developed later.

Pretreatments of flower heads, buds or excised anthers have been shown to substantially increase yields in some species. In *B. juncea* (Sharma & Bhojwani 1985) culture of anthers for 1-5 days at 35°C or for 3 days at 5°C stimulates embryogenesis. In *B. oleracea*, Ockendon (1984) found that 16 hours at 35°C was the optimum treatment for excised anthers, but thermal shock at 30°C was ineffective. Keller & Armstrong found 1-3 days at 35°C to be effective in *B. napus* (1978), *B. campestris* (1979), *B. oleracea* var *acephala* (1981) and *B. oleracea* var *italica* (1983). It is not clear how high temperature acts to stimulate embryogenesis but it has been suggested that it may cause dissolution of the microtubules leading to abnormal division of the microspore nucleus (Hepler & Palevitz 1974).

Culture medium is often considered to be of prime importance in culture protocols. Dunwell (1985b) believes that it is less important than the variables of growth conditions and pollen stage described earlier. It is known that agar contains substances that are deleterious to pollen survival in culture (Dunwell 1985b). It is recommended that alternative gelling agents should be used, such as agarose or Gelrite, or that liquid medium should be used. Liquid medium has drawbacks in that anthers or microspore calli can sink and fail to develop further under these anaerobic conditions. Ficoll at $200gdm^{-3}$ (Kao 1981) has been used in barley to increase buoyancy of calli.

There is little analytical information available on the mineral salt and vitamin components in the media, but media based on formulation of Murashige and Skoog (1962) or on the B_5 medium of Gamborg *et al* (1968) have been widely used (Dunwell 1985b). Dunwell (1985 pers. comm.) believes that salt and vitamin components are some of the least important influences on anther culture.

Species can be roughly separated into two groups ie those requiring low sucrose levels (2-4%) and those requiring high sucrose levels (5-15%). This seems to be related to whether pollens are shed in the bicellular state e.g. *Solanaceae*, or tricellular state e.g. *Cruciferae*, *Graminae*. The former group require

low sucrose and the latter high sucrose. This is not just an osmotic response, as substitution of other sugars for sucrose does not result in the same stimulatory effect (Keller *et al* 1975). It is believed that sucrose inhibits growth of diploid anther wall and filament tissues and also specifically induces division in pollen. There is some evidence that differences in genotypic response are overcome to some extent by use of a very high level of sucrose (16%). This is approximately equivalent to the osmotic pressure of the supernatant of anther homogenates (Dunwell & Thurling 1985).

Species can also be separated into two groups on the basis of PGR requirements ie PGR-independent species (e.g. *Solanaceae*) and PGR-dependent species (e.g. *Graminae, Cruciferae*). However there is some evidence (Dunwell 1985b) that under optimal conditions some response is possible from the latter group if anthers are grown on PGR-free medium. It seems that for the PGR-dependent species the particular concentrations and combinations of PGRs are relatively unimportant (Dunwell 1985b). Maheshwari *et al* (1982) point out that in about 80% of reports of successful anther culture PGRs are included in the medium. In the *Cruciferae* 2,4D is the auxin usually supplied (Dunwell 1985a) though NAA and IAA have also been used. Kinetin and BA are sometimes supplied in combination with an auxin. The high sucrose levels needed by the

Cruciferae allow use of high auxin levels which would otherwise stimulate proliferation of the somatic tissues (Sunderland & Dunwell 1977).

A number of other aspects of culture conditions have been shown to be important in some species, such as culture vessel atmosphere in *Nicotiana tabacum* (Dunwell 1979) and anther orientation in barley (Shannon *et al* 1985) and rice (Mercy & Zapata 1987) but no clear guidelines have emerged for the *Cruciferae*.

A number of workers have investigated the induction of haploids by culture of isolated pollen. This potentially has a number of advantages (Bajaj 1983):

1. The possibility of embryos or callus arising from somatic tissues is eliminated.

2. Any uncontrolled influences from the anther wall are removed and this should lead to a better understanding of the hormonal control of androgenesis as chemicals can be incorporated into the medium and tested more directly.

3. It has been suggested that the confined conditions within the anther lead to reduced yield as a result of competition between pollen grains, so that isolation of the pollen should give a higher yield as this competition would be reduced.

4. Pollen grains are more suitable for use in mutation or genetic transformation studies.

Most early experiments did not show significant increases in embryo formation over anther culture techniques. These often involved preculture of pollen within the anther or use of the anther as nurse tissue. Lichter (1982) first demonstrated an increase in yield of embryos in isolated microspore culture of *Brassica napus*. Chuong & Beversdorf (1985) found a high frequency response in isolated microspores of *B. napus* and *B. carinata* which was influenced by genotype and medium. Swanson *et al* (1987) improved techniques for isolation of the pollen and obtained yields of 700-1000 embryos per bud of *B. napus* which were considerably higher than reported in most anther cultures e.g. Ockendon (1984), Loh & Ingram (1982). Particular advantages of this technique were reduction in time and ease of handling very small flower buds. The necessity for PGRs in the medium also seemed to be removed.

An alternative method for haploid induction is by ovary or ovule culture where the female megaspore is induced to develop into an entire plant. This may occur by callus production, by embryoid formation or by direct shooting. This was first reported in barley in 1976 (San-Noeum) and has since been extended to several species. The technique is subject to many of the same problems as in anther culture such as the influence of

genotype and the problem of distinguishing callus or embryoids arising from somatic tissue. One advantage appears to be that albinos are much less frequent than in anther derived plantlets (Yang & Zhou 1982). It may be a useful approach for production of haploids in species where anther culture has failed, but currently responses are too low and limited to be of much practical application.

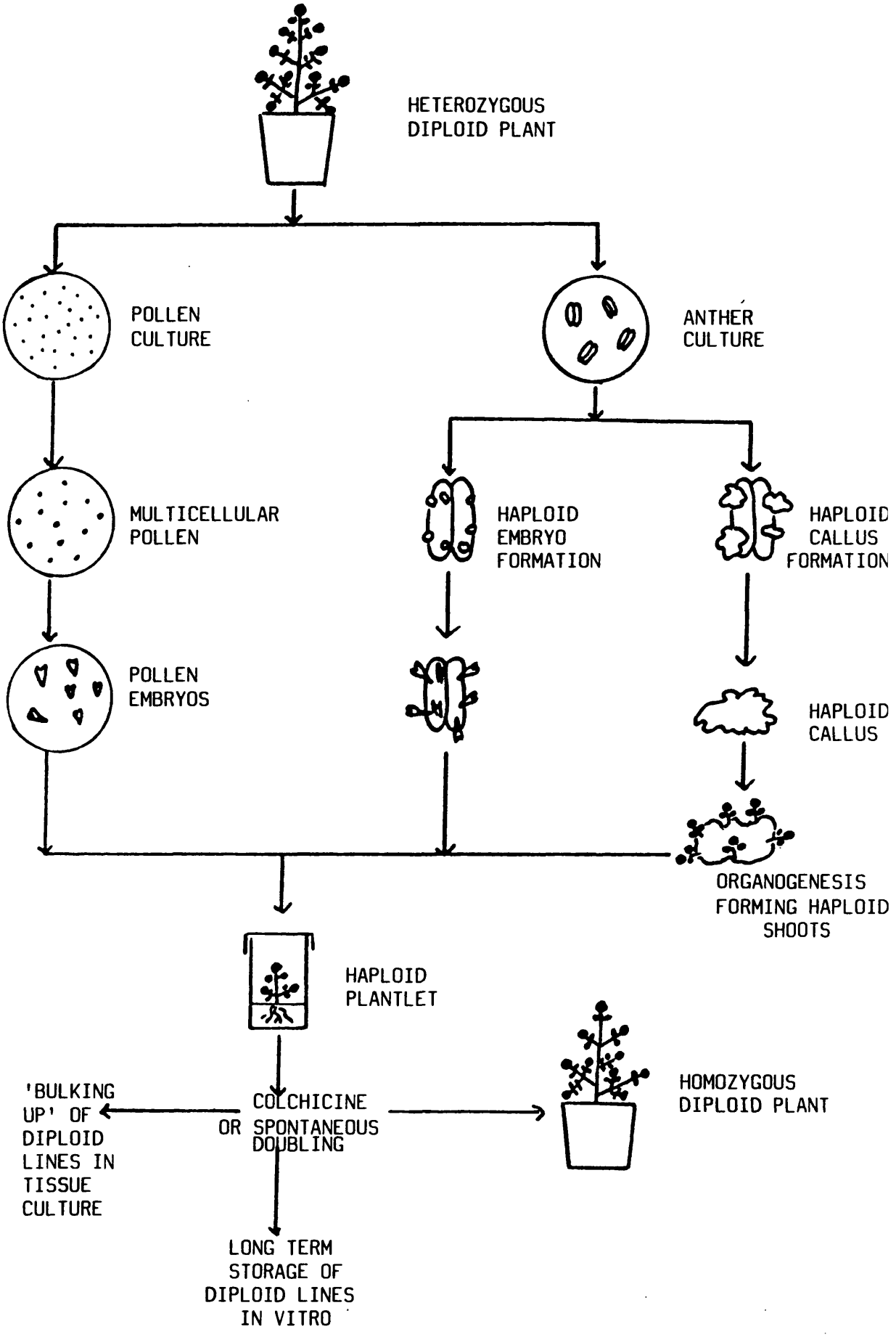
In the last decade intensive work has been started in many countries to utilize doubled haploid lines obtained from pollen. Three varieties of rice, two of wheat and three of tobacco have been released in China (Bajaj 1983). In Japan, a tobacco variety resistant to bacterial wilt has been obtained through anther culture (Maheshwari *et al* 1982). In the U.K. anther culture techniques are being used only in breeding programmes with oilseed rape while in Canada anther-derived doubled haploid lines of oilseed rape are under field test and lines showing potential yield improvements have been selected (Keller *et al* 1983).

The great potential of haploids in crop improvement can be seen, provided that a technique for their production at sufficiently high frequency can be developed. Nitsch (1983) believes that despite difficulties most species that have been tried respond positively and that it is not too optimistic to think that androgenesis is feasible with any species. The

positive results in other members of the *Cruciferae* suggest that haploid production from microspores should be possible in *Rorippa nasturtium - aquaticum*.

The experimental procedures and results of anther, pollen and ovule culture are described in sections 3.2, 3.3 and 3.4, and their significance is discussed in sections 4.2, 4.3 and 4.4.

SUMMARY OF MAIN MODES FOR HAPLOID PRODUCTION FROM POLLEN GRAINS



1.3 Long Term Storage for Regenerated Material

Future plant breeding programmes will be increasingly dependent on extensive germplasm collections as primitive cultivars and related wild species disappear as a result of changes in agricultural practice. Many species can be stored as seed which requires little space or attention, but this is not suitable for all crops, for example in outbreeding or heterozygous species where it is necessary to store exact genotypes (Henshaw *et al* 1980a). In a relatively unimproved crop like watercress it is important to approach crop improvement from a wide base of genotypes (Johnson 1974) particularly as genotype is the most important factor in determining the success of anther culture (Dunwell 1985a) so a method for maintaining such a wide range of genotypes could have important applications.

In vitro techniques can offer considerable advantages over traditional methods involving regular periods of vegetative growth during which losses can occur from pest or pathogen attack, natural disasters, human error etc. Other advantages include saving of space and high multiplication rates when required (Henshaw *et al* 1980a).

An *in vitro* system with high multiplication rates is not ideal for germplasm storage as it requires frequent

attention and high cell division rates are likely to be correlated with high mutation rates (Henshaw *et al* 1980b).

Ideally cell division would be completely suppressed and this has been achieved in a few cases by use of ultra low temperatures. Several species of *Solanum* have been frozen in liquid nitrogen at -196°C and thawed with acceptable survival rates (Henshaw *et al* 1980a). It is not yet known whether these techniques will prove to be generally applicable. Techniques based on storage of cultures under conditions which permit only minimal rates of growth are likely to be more widely employed. Such methods have the advantage that stored material is readily available for use and stocks are replenished as the cultures grow (Henshaw *et al* 1980b).

Not all types of culture system can be considered suitable for germplasm storage. Callus and cell suspension cultures in particular are associated with genetic instability and loss of morphogenetic potential (Gamborg and Shyluk 1981). Commercial experience with shoot tip cultures suggests that such cultures. particularly those in which multiplication is based on non-adventitious shoot production usually retain the genetic stability that is characteristic of the shoot apical meristem *in vivo* (Murashige 1974).

Reduced growth rates can be achieved either by use of specific growth inhibitors or by use of conditions which affect the metabolism in a more general way e.g. temperature, gaseous conditions, nutrient availability, osmotic stress. Mullin and Schlegel (1976) showed that *in vitro* grown strawberries could be maintained at 4°C for up to six years. Lundergan and Janick (1979) indicated that survival in apple shoot tips could be increased considerably for up to one year at temperatures of 1°C and 4°C in the dark compared to controls at 26°C in the light. Low temperature treatments have also been shown to be successful in *Solanum* species (Westcott *et al* 1977).

However low temperature facilities are not always available for storage of large numbers of cultures so other methods of limiting growth have been investigated. Addition of mannitol at 1%, 3% and 6% to increase osmotic pressure has increased survival in *Solanum* shoot tip cultures (Henshaw *et al* 1980a, Westcott 1981b). Cultures varied in their responses to mannitol but shoots were usually stunted with short internodes, poor root development and chlorotic leaves. Longevity is probably increased by slowing growth, so that the time before medium components become limiting is increased. Abscisic acid (ABA) probably acts by a similar mechanism, but as a hormonal growth inhibitor. It has been used successfully at rates of 5 to 20mgdm^{-3} in *Solanum* species (Westcott *et al* 1977, Henshaw *et al*

1980a). Medium enrichment by addition of up to 8% sucrose; increase in medium volume and use of polypropylene film to allow gas exchange without desiccation have all been shown to increase longevity of cultures (Henshaw *et al* 1980b) particularly in combination with a lowered temperature. Chemical growth retardants have also been tested in *Solanum* species. B995, Phosphon D and maleic hydrazide all showed slightly increased survival in treated cultures over control cultures (Westcott 1981b).

In view of the success of methods that affect the metabolism in a general way and the fact that most of these can be carried out with readily available chemicals and facilities, these would seem to be most useful for application to other species. Reduced growth rate storage *in vitro* would seem to be feasible and certainly has potential applications for storage of exact genotypes of regenerated plants of watercress.

The experimental procedure and the results are presented in section 3.5 and their significance is discussed in section 4.5.

2. MATERIALS AND METHODS

2.1 PLANT MATERIAL

Growth and flowering of parent plants

A number of seeds of various watercress strains were sown onto Levington universal compost in covered pots to maintain high humidity. After 2 weeks seedlings were selected at random to be grown on individually. Cuttings were taken from each seedling to produce clonal lines of each plant. In watercress adventitious roots form readily at the nodes and these will root into compost very rapidly. Cuttings were all placed in Levington universal compost in 9cm pots. Initially they were rooted under mist in a glasshouse but problems with pests made this unsatisfactory and cuttings were later rooted in a growth cabinet at 20°C and a 20 hour photoperiod.

Plants were maintained in two growth cabinets made by Fisons and Saxcil, at the same light intensity (10 Wm^{-2} and 20°C and a 20 hour photoperiod. Strict sanitation was carried out to limit the entry of pests such as greenfly, red spider mite and cyclamen mite, as infestations proved to be inhibitory to flowering. Pirimor was used to control greenfly, Pynosect and then biological control by *Phytoseleius* were used against red spider mite. Cyclamen mite proved to be particularly troublesome, as the position of the growth cabinets prevented use of chemicals that would

eradicate it. It proved possible to control it by increasing the cabinet temperature to 50°C for 12 hours and then using clean material from seed or tissue culture.

Seeds were all supplied by Dr S. Rothwell of Hampshire Watercress Ltd. and Mr J. Watts of Sylvasprings Ltd., and the following clonal lines of each strain were raised.

English Dark Green	5 lines	(A to E)
Japanese	11 lines	(J1 to J11)
Australian	2 lines	(Aus1, Aus2)
Californian	2 lines	(USA1. USA2)
New Zealand	2 lines	(NZ1, NZ2)

There were visible differences between lines within a strain in characters such as leaf shape, colour and flowering time but these were not quantified. Flower buds were usually initiated between 3 and 5 weeks from cuttings, though 2 clones of English watercress were recalcitrant and could not be induced to flower. Flower initiation was also tested at 15°C for 3 lines of English watercress and while this inhibited red spider mite, flowering was also inhibited.

2.2 STERILIZATION OF BUD MATERIAL

Several techniques for sterilization of bud material were tested. The method chosen was using individual buds which were placed in stirred 70% ethanol for 20 seconds and then in stirred sodium hypchlorite solution with 2% available chlorine (w/v) and containing a few drops of Gurr 7X as a wetting agent for 30 seconds. This gave 95% uncontaminated buds when tested on Lab M "Lab 8" nutrient agar. This was also the shortest of the treatments tested which should minimize damage caused by exposure to the sterilizing solutions. Prolonged sterilization treatments can adversely affect the response of anthers in culture (Dunwell 1985b).

Anthers from the buds were then tested for viability using tetrazolium chloride solution (section 2.3) and were found to be viable.

2.3 VIABILITY

Viability of anthers was tested using a 0.5% (w/v) aqueous solution of tetrazolium chloride. This is a colourless soluble salt which is reduced by electrons from aerobic respiration to form triphenyl formazan which is insoluble and red in colour. The rate of formation of the red colour is directly proportional to the rate of respiration of cells. Anthers were immersed in the solution for two hours in the dark and

then examined microscopically for formation of the red colour.

2.4 STERILIZATION OF NODAL MATERIAL

Lengths of stem of 80-120mm were collected and the leaves removed. They were placed in stirred 70% ethanol for 1 minute and then in stirred sodium hypochlorite (2% available chlorine) with a few drops of wetting agent (Gurr 7X) for 3 minutes (Wainwright and Marsh 1986). They were rinsed three times in sterile distilled water and then dissected into nodal cuttings under aseptic conditions.

2.5 MEDIA

PGRs were all supplied by Sigma, except Dropp (thidiazuron) which was donated by Schering AG Ltd. and Paclobutrazol which was used in the form of "Bonzi" made by ICI. Other salts, vitamins and organics were supplied by Sigma, BDH or Fisons and were analytical grade wherever possible.

Experiments were carried out on MS (Murashige and Skoog 1962) medium, unless otherwise stated. Components of media are given in Appendix A. Stock solutions were made up and stored at 4°C. Solutions of plant growth regulators (PGRs) at 10^{-3}M were also stored at 4°C. Sucrose was used as the carbon source at 2% w/v unless

otherwise stated. PGRs if used were added and the pH adjusted to 5.7±0.2 with 1M NaOH or 0.5M HCl before addition of Lab M "MC 2" agar at a rate of $7gdm^{-3}$. Media were sterilised by autoclaving at 121°C for 15 minutes.

After cooling to about 50°C media were dispensed into 50mm deep form "Sterilin" plastic petri dishes in volumes of 15ml per dish or 100ml "Sterilin" screw topped plastic jars in volumes of 25ml per jar. All petri dishes were sealed with ethanol sterilised "Parafilm R".

2.6 CULTURE CONDITIONS

Incubation conditions were 16 hours photoperiod at 10 Wm^{-2} from cool white fluorescent tubes and 25°C in a growth room unless otherwise stated.

2.7 CULTURE OF *IN VITRO* DONOR MATERIAL

A single sterile nodal cutting was placed on PGR-free MS medium in a 50mm petri dish, and incubated as above. Cultures were maintained by subculture of shoot tips every 4-6 weeks onto the same media. Use of small petri dishes gave multiple shoots without use of PGRs which may have mutagenic effects (Turkula and Jalal 1985).

2.8 WEANING OF TISSUE CULTURED SHOOTS

A 2:1 mixture of Levington universal compost and Silvaperl perlite was used in 150mm x 210mm trays. Tissue cultured shoots 20-40mm in length were transferred into the compost. The compost was kept damp by standing the tray in water and it was covered with a rigid plastic lid. The plastic lid was gradually ventilated and was removed completely after 7 days. Incubation was carried out at 20°C during a 16 hour photoperiod and at 15°C during an 8 hour dark period.

3. RESULTS

3.1 REGENERATION FROM CALLUS

Four experiments on shoot regeneration from callus were carried out.

Donor plant material was obtained from nodal cultures of clonal line A of English Dark Green Watercress grown as described in section 2.7. Explants were taken after 4-6 weeks of culture. Use of *in vitro* material overcame the seasonal variations seen in field grown material (Clare & Collin 1974). All experiments were set up with five explants per plate and seven replicate plates per treatment. Raw data are given in Appendix B.

Summary of regeneration from callus

Exp. 1 Effect of cytokinin and auxin on shoot regeneration. Table 1. Figs. 1a, 1b & 2.

Exp. 2 Influence of some culture variables (gelling agent, light/dark treatments) on shoot regeneration. Tables 2, 3 & 4. Figs. 3, 4, 5a, 5b & 6.

Exp. 3 Effect of different explants (leaf and petiole) on shoot regeneration.
Table 5.

Exp. 4 Effect of genotype on shoot regeneration.

Table 6.

Exp. 1. Effect of cytokinin and auxin on shoot regeneration

To investigate the effect of cytokinin and auxin on shoot regeneration, a fully replicated experiment was set up using 4 levels of each PGR i.e. 0, 0.5, 2.5 and 12.5μM. The PGRs tested were Dropp and BA as cytokinins; with NAA and 2,4D as auxins. Petiole explants (2-4mm long) were taken aseptically from donor cultures of clone A. Five explants were placed on each plate orientated so that it was clear which end had been proximal to the stem. Initially cultures were placed in the dark for 4 weeks on media with a cytokinin and an auxin and were then subcultured onto media containing the same levels of cytokinin but no auxin. Subsequent subcultures at 4 weekly intervals were onto the same media. These were placed in a 16 hour day photoperiod. Shoot formation was scored every 4 weeks and any shoots were carefully removed and placed on PGR-free medium.

Results

Control explants on PGR-free medium showed only occasional root formation, while explants on media with an auxin only showed copious root formation, but no

callus formation. Callus formed on all explants where a cytokinin was present. This was dark green and very compact where there was no auxin, and formed only at the end of the explant which had been proximal to the stem. Calli became larger and less compact with increasing concentrations of PGRs and formed from the whole explant. There was little further formation of roots once auxin had been removed from the medium, though calli continued to grow. Shoot formation only occurred from callus.

Table 1 shows numbers of shoots formed on various concentrations of cytokinin and auxin. The highest frequency of shoot production was at 12.5μM for Dropp and 0.5μM for 2,4D.

The numbers of shoots formed per treatment were analysed by anovar. Missing data, where replications had been lost due to contamination, were replaced by using the missing value formula given by Bishop (1980). The results of the analysis showed that the concentrations of auxin and cytokinin and the interaction between them were significant at the P = 0.1% level. The data from Table 1 have been used in Fig 1 to show the effect of each cytokinin concentration on the number of shoots produced over the full range of auxin concentrations and conversely the effect of each auxin concentration over the full range of cytokinin concentrations. Fig. 1a shows the effect of cytokinin on shoot regeneration and Fig. 1b shows the effect of auxin on shoot regeneration and it can be seen that

(1) Dropp stimulated the production of more shoots than BA at all concentrations.

(2) The number of shoots increased with increasing concentrations of Dropp.

(3) 2,4D was better than NAA for shoot production except at 12.5μM.

(4) The number of shoots decreased with increasing concentrations of 2,4D above 0.5μM.

(5) Occasional shoots formed on 0μM auxin, but shoots did not form on 0μM cytokinin.

Fig. 2 shows change in regeneration potential with time for various combinations of PGR. It can be seen that shoots were produced at different times depending on the treatment.

(1) For Dropp and NAA there was a peak in shoot production at week 4 with all calli becoming necrotic by week 20.

(2) BA and NAA gave a slight peak at 12 weeks.

(3) BA and 2.4D gave longer lived calli with shoot production ceasing at 28 weeks.

(4) Dropp and 2,4D gave the most long lived calli with a large peak in shoot production at 28 weeks.

TABLE 1. Effect of cytokinin and auxin on shoot yield over nine subcultures (36 weeks) n = 35

shoot yield = N° of shoots produced

AUXIN LEVEL µM		CYTOKININ LEVEL µM								
		DROPP	0.0	0.5	2.5	12.5	0.0	0.0	0.0	0.0	Total
NAA	2,4D	BA	0.0	0.0	0.0	0.0	0.0	0.5	2.5	12.5	
0.0	0.0		0	0	0	1	0	0	0	0	1
0.5	0.0		0	4	0	1	0	1	0	0	6
2.5	0.0		0	4	2	5	0	0	5	1	17
12.5	0.0		0	3	0	4	0	0	5	3	15
0.0	0.0		0	1	0	0	0	0	3	0	4
0.0	0.5		0	3	17	38	0	0	0	4	62
0.0	2.5		0	4	18	4	0	0	0	3	29
0.0	12.5		0	1	0	4	0	0	2	1	8
Total			0	20	37	57	0	1	15	12	142

Note: The treatments that involved 0µM auxin and Dropp and 0µM auxin and BA were both carried out twice on separate occasions and it can be seen that reproducibility is not 100%.

Fig. 1a: Effect of cytokinin on shoot yield

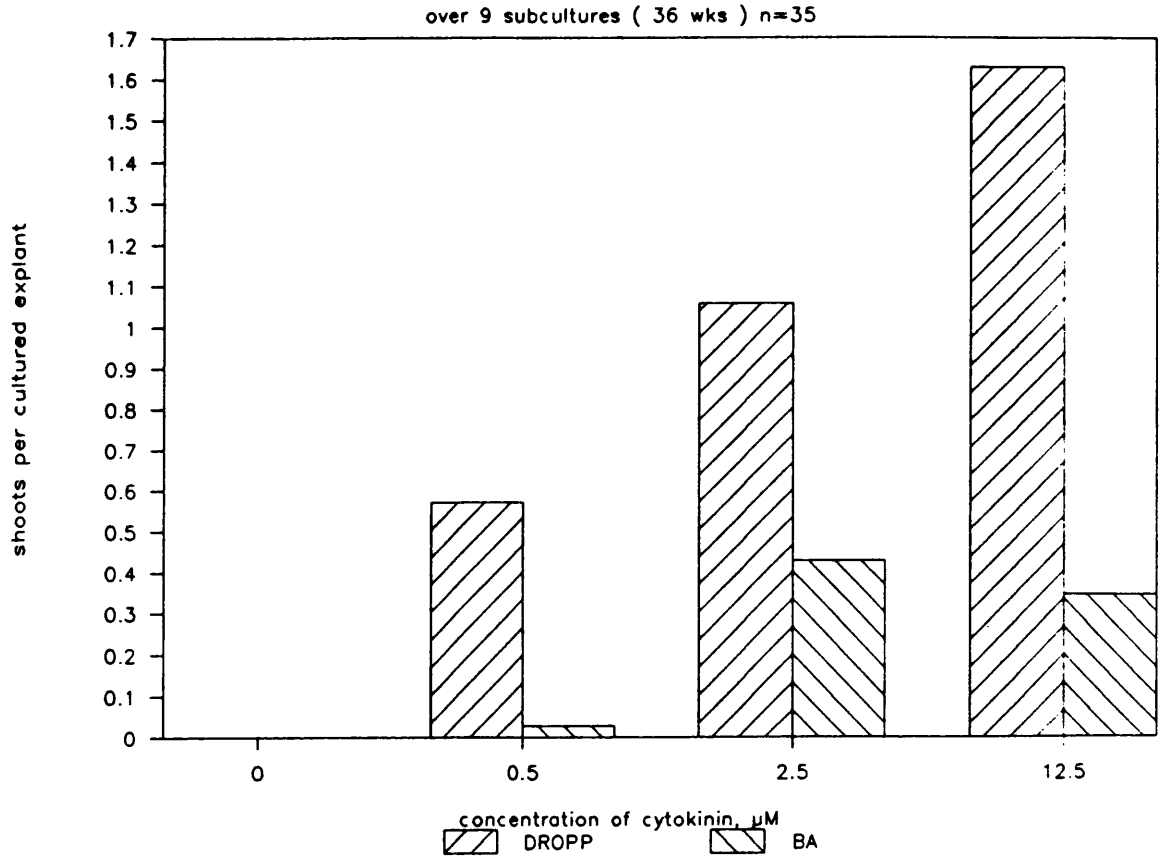


Fig. 1b: Effect of auxin on shoot yield

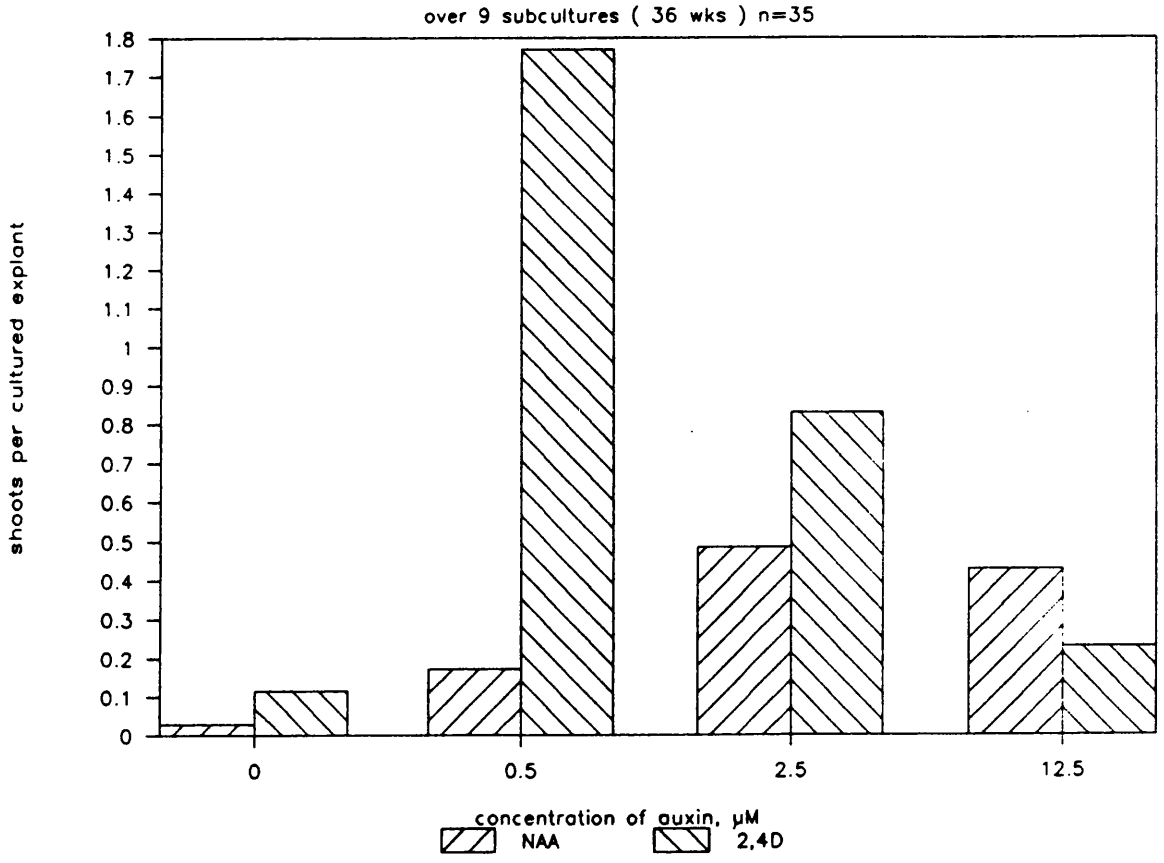
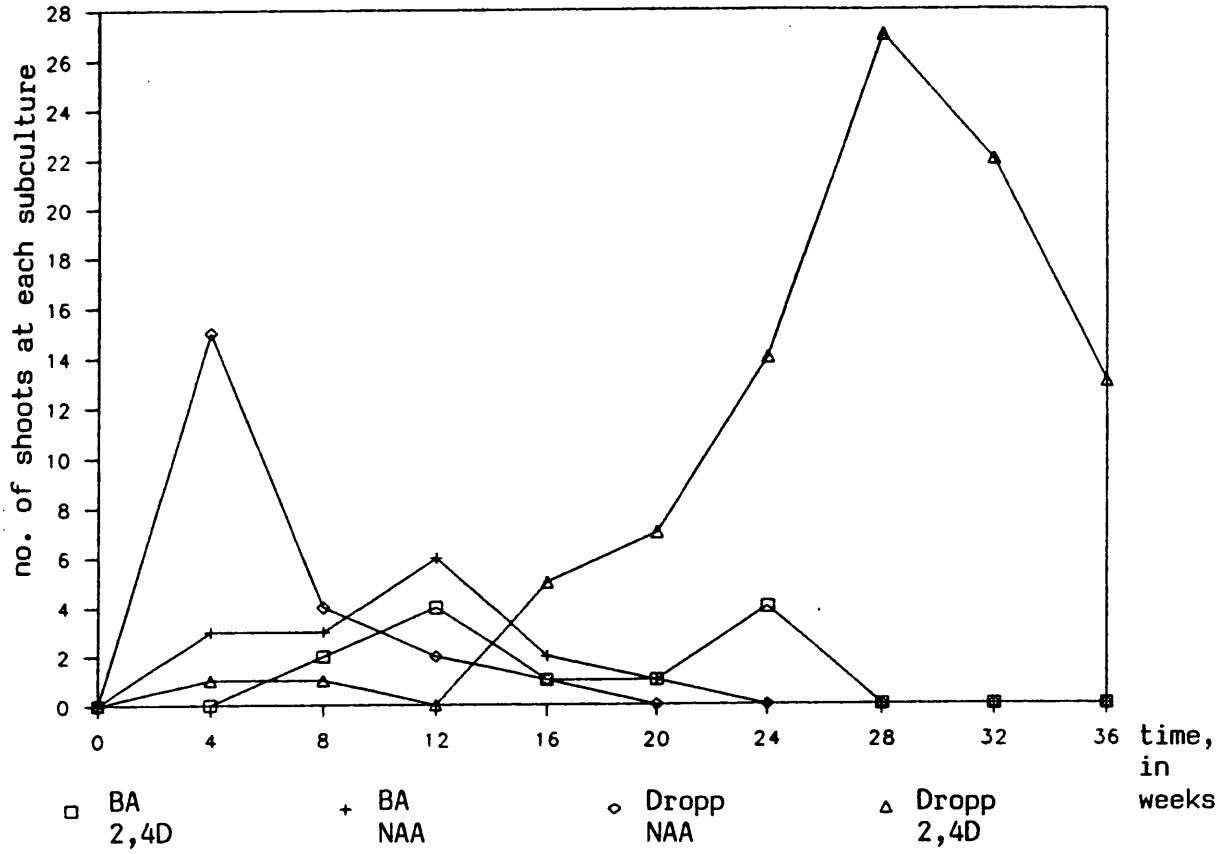


FIG. 2. Effect of cytokinin and auxin on change in regeneration with time in culture.



Exp. 1. Effect of cytokinin and auxin on shoot regeneration

1a shoot regeneration from callus formed on 0.5µM Dropp and 2.5µM NAA x 2.2

1b shoot regeneration from callus formed on 2.5µM Dropp and 12.5µM NAA x 2.2

Exp. 2 Influence of some culture variables on shoot regeneration

To investigate the influence of some culture variables on organogenesis, petiole explants were excised as described above and treated in a number of ways which are shown in table 2, except in treatment (Trt.) 10 where the explants were leaf sections. Gelrite was supplied by Kelco, agar was Lab M and casein hydrolysate (CH) was enzymatic. Each treatment was tested with 3 levels of Dropp i.e. 0.5, 2.5 and 12.5μM and NAA was included in the medium at 2.5μM for the first 4 weeks of culture. Illumination was ~~either dark or~~ light with a 16 hour photoperiod or dark. Subculture onto fresh medium with Dropp only, unless otherwise stated, was carried out every 4 weeks, and shoot formation was scored.

Results

There were differences between the appearance of callus produced on various treatments. Trt. 1 (Gelrite) gave the largest and darkest green callus, while Trt. 8 (+ NAA) gave pale friable callus. Trts. 4 and 5 (CH) initially gave very small calli and Trts. 7 (light) and 10 (leaf sections) gave globular calli. Calli in the dark (Trt. 6) and on Trts. 4 and 5 were longest lived to 28 weeks, while other explants on other treatments had become necrotic at 20-24 weeks.

The number of shoots produced are shown in Table 3 and the number of productive explants in Table 4 and these are also shown in Fig. 3.

Both number of shoots produced and number of productive explants were analysed by χ^2 with Yates correction as the data were discontinuous (Goldstein 1964). For number of shoots produced there was a significant effect of the culture variables and further analysis by χ^2 indicated that the significant treatments were

Trt. No.	Treatment	Level of Significance
7	light	P = 0.1%
4	CH at 250mgdm^{-3}	P = 0.1%
1	Gelrite	P = 1%
9	PGR-free	P = 1%

However it can be seen from Fig. 4 that not all were better at the same concentration of Dropp.

Fig. 5a shows that there was a significant difference (P = 5%) between shoot production on 0.5μM Dropp and the higher concentrations, but it can be seen from Fig. 4 that the data from Trt. 7 dominate the results.

Fig. 5b shows that there is an increase in number of shoots per productive explant with increasing concentration of Dropp, though there was a slight decrease in number of explants responding at 12.5μM Dropp.

Fig. 6 shows percentage explant response and the analysis indicated that the only significant effect was light (Trt. 7) which was significant at the P = 0.1% level.

TABLE 2. Exp 2 - Influence of some culture variables on shoot regeneration - experimental layout.

TRT. NO.	FIRST TREATMENT gelling agent	dark/ light	additions	SUBSEQUENT TREATMENTS gelling agent	dark/ light	additions/ subtractions
1	Gelrite 2gdm^{-3}	dark	-	Gelrite	light	-
2	MC2 agar 7gdm^{-3}	dark	-	MC2 agar	light	-
3	MC29 agar 7gdm^{-3}	dark	-	MC29 agar	light	-
4	"	dark	250mgdm^{-3}CH	"	light	no CH
5	"	dark	500mgdm^{-3}CH	"	light	no CH
6	"	dark	-	"	dark	-
7	"	light	-	"	light	-
8	"	dark	-	"	light	+2.5µM NAA
9	"	dark	-	"	light	no Dropp
10	"	dark	-	"	light	-

n = 35
light = 16 hours 10WM^{-2}
PGRs = 2.5µM NAA for first 4 weeks
0.5, 2.5 or 12.5µM Dropp throughout

EXP. TWO - Influence of some culture variables on shoot regeneration.

TABLE 3. Total no. of shoots produced over 7 subcultures (28 weeks) n = 35.

Trt. No.		1	2	3	4	5	6	7	8	9	10	Total No. of shoots
DROPP	0.5	12	4	5	2	1	0	13	2	1	0	40
LEVEL	2.5	5	2	2	8	4	1	23	2	10	6	63
µM	12.5	2	7	4	16	3	5	15	1	8	1	62
Total		19	13	11	26	8	6	51	5	19	7	165

TABLE 4. Total no. of productive explants over 7 subcultures (28 weeks) n = 35.

Trt. No.		1	2	3	4	5	6	7	8	9	10	Total No.
DROPP	0.5	5	4	3	2	1	0	7	2	1	0	25
LEVEL	2.5	3	2	1	4	3	1	11	1	5	4	35
µM	12.5	1	3	3	4	3	4	7	1	4	1	31
TOTAL		9	9	7	10	7	5	25	4	10	5	91

FIG. 3. Influence of some culture variables on shoot yield and number of productive explants over 7 subcultures. n= 35

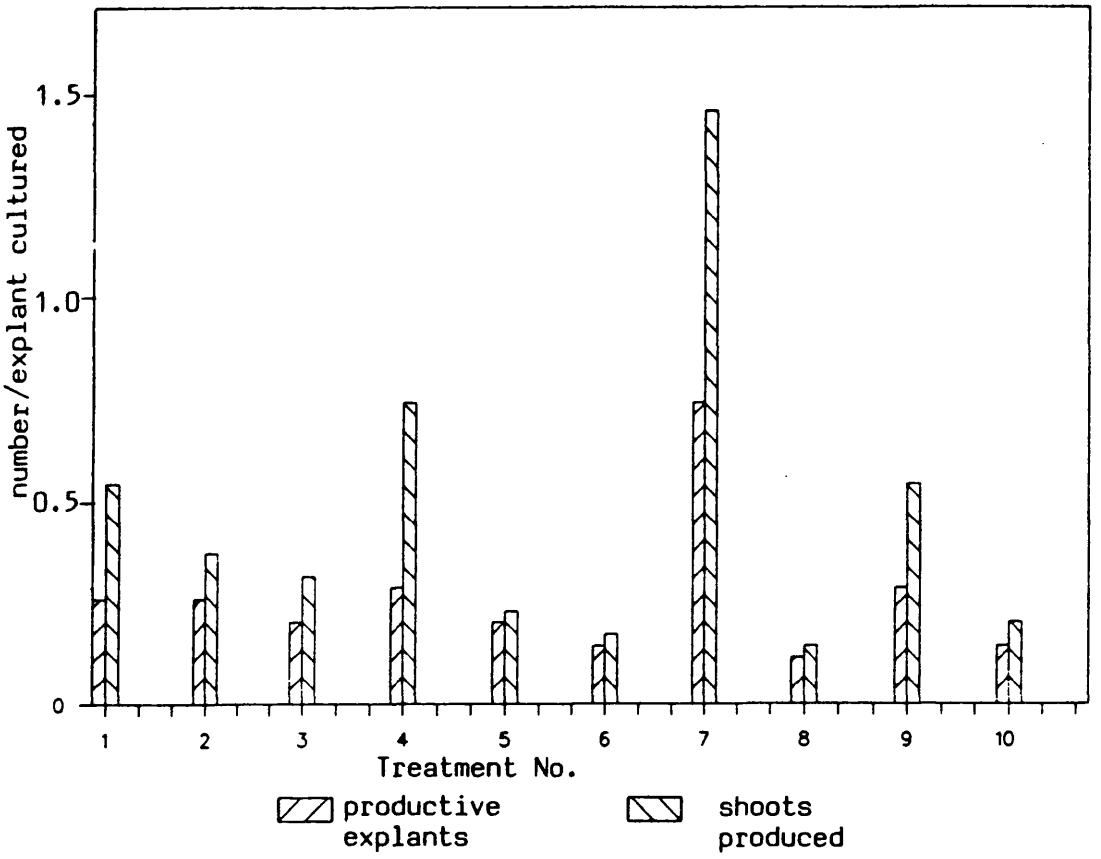


FIG. 4. Influence of some culture variables on shoot yield at 3 concentrations of Dropp over 7 subcultures. n = 35

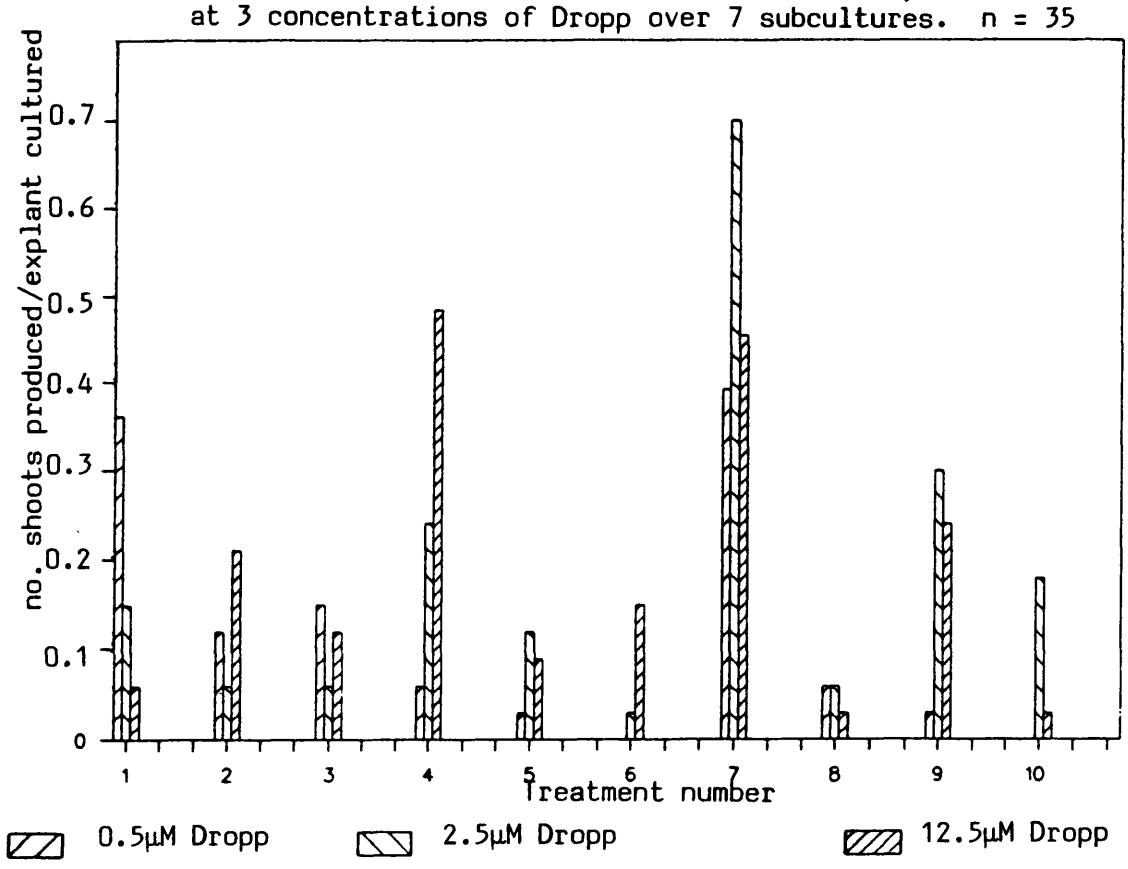


Fig 5a Influence of culture variables

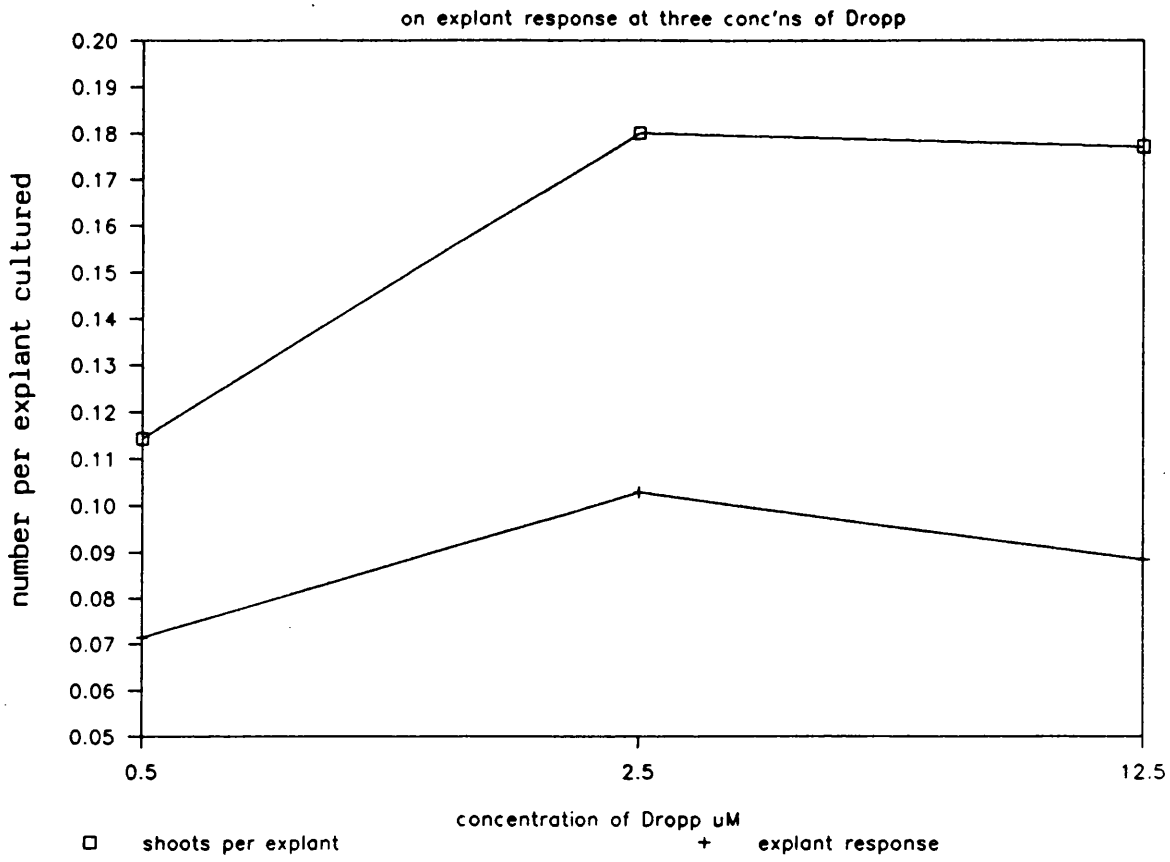
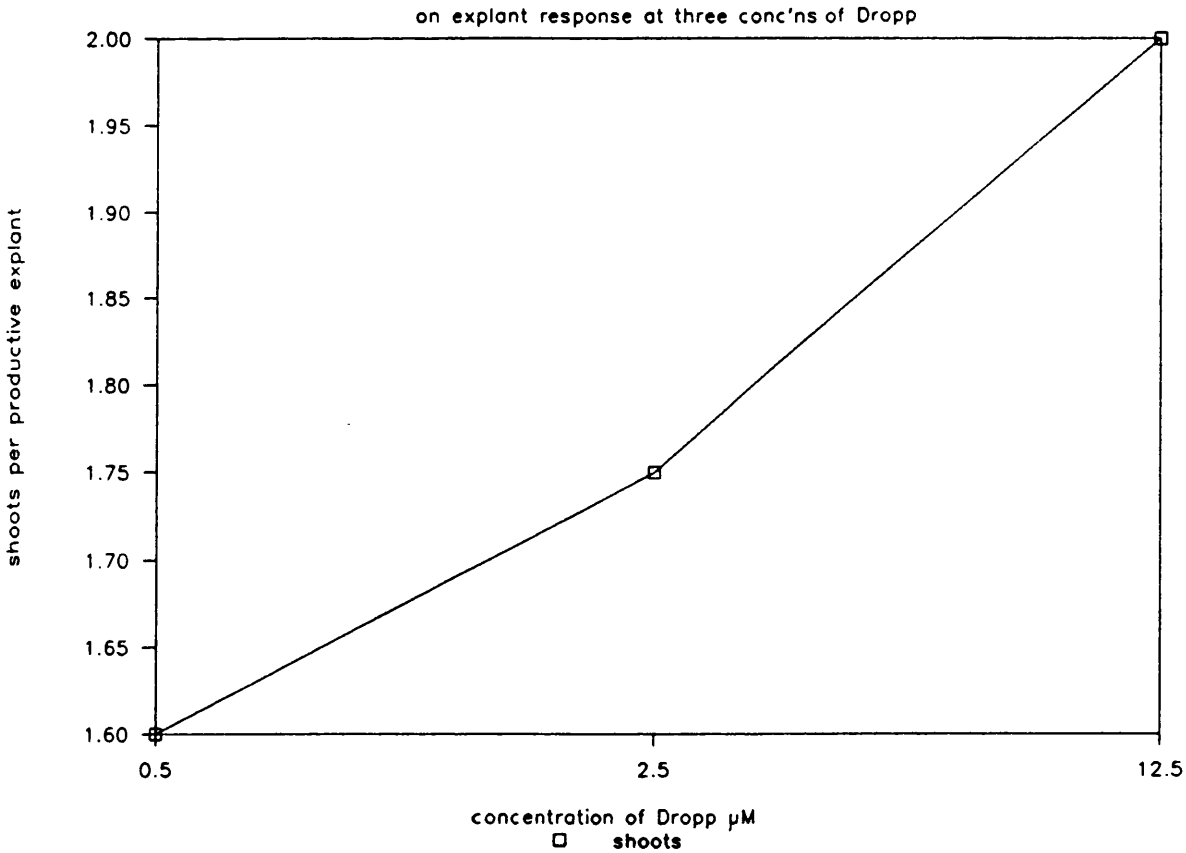
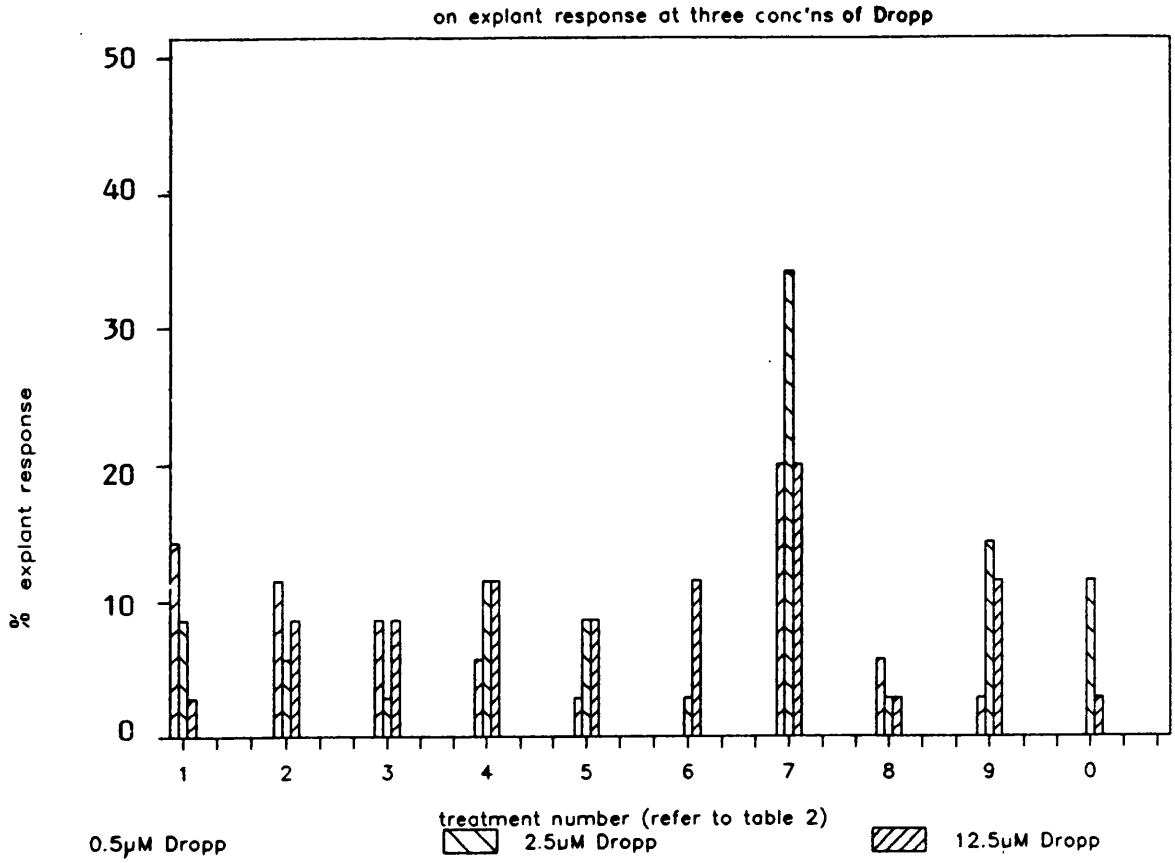


Fig 5b. Influence of culture variables

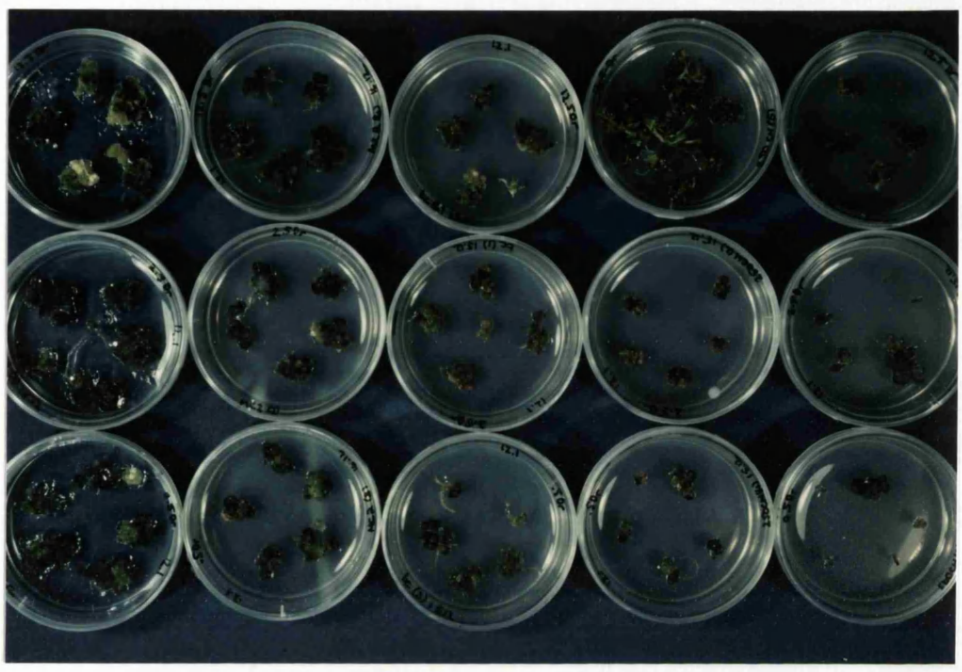


explant response = N° of explants producing shoots.

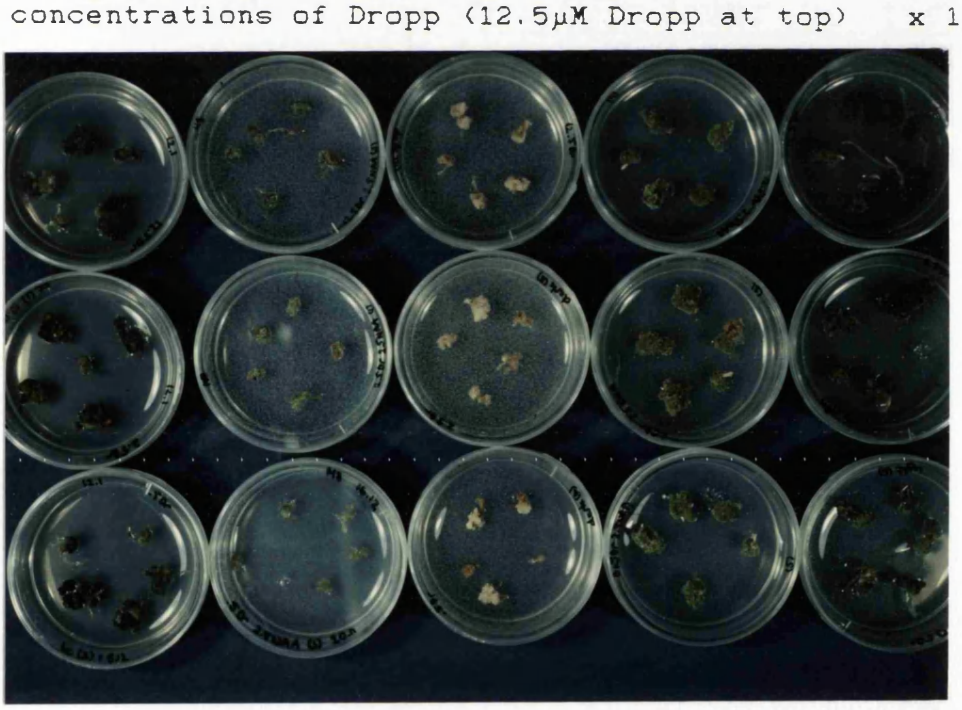
Fig 6. Influence of culture variables



Exp. 2. Influence of some culture variables on shoot regeneration



2a effect of Gelrite, MC2 agar, MC29 agar 250mgdm^{-3}CH, 500mgdm^{-3}CH on callus formation at three concentrations of Dropp (12.5µM Dropp at top) x 1



2b Effect of MC29 agar, subculture to PGR free medium, culture in the dark, continued culture on 2.5µM NAA and 16 hours photoperiod on callus formation at 3 concentrations of Dropp (12.5µM Dropp at top) x 1

Exp. 3. Effect of type of explant on shoot regeneration.

An experiment was set up to compare the effect of different explants i.e. leaves and petioles. The PGRs used were BA and 2,4D at 0, 0.5, 2.5 and 12.5μM. Petiole explants were as described above and leaf sections were approximately 2-3mm x 3-4mm. Subculture was onto media containing the same levels of cytokinin but no auxin every four weeks and shoot formation was also scored.

Results

The results of Exp. 3 are presented in Table 5 and indicate that there is a difference in response between leaf and petiole explants and only four shoots on leaf explants. Shoot numbers were too small for a valid statistical analysis.

TABLE 5 Total no. of shoots produced on leaf or petiole explants of Clone A on BA + 2,4D over 7 subcultures (28 weeks)

BA µM / 2,4D level µM	0 leaf/petiole		0.5 leaf/petiole		2.5 leaf/petiole		12.5 leaf/petiole	
0.0	0	0	0	0	0	3	0	0
0.5	0	0	0	0	0	0	0	4
2.5	0	0	0	0	0	0	0	3
12.5	0	0	0	0	0	2	4	1

Exp. 4. Effect of genotype on shoot regeneration

An experiment was also set up to compare the response of two different genotypes. These were Clone A and the clonal line of Japanese watercress labelled J1. The explants used were petiole sections as described above. The media contained PGRs BA and NAA at 0, 0.5, 2.5 and 12.5μM. Subculture was carried out every 4 weeks onto media with the same levels of cytokinin but no auxin and shoot formation was scored.

Results

It can be seen from Table 6 that there is a difference in response between the two genotypes. Sixteen shoots were produced on explants on Clone A while only three shoots were produced on explants of Clone J1. The highest number of shoots was produced at 2.5μM BA from Clone A. However the positive results were too few for a meaningful statistical analysis.

TABLE 6 No. of shoots produced on petiole explants of Clone A and Clone J1 on BA + NAA over 7 subcultures (28 weeks) n = 35

BA level µM	0		0.5		2.5		12.5	
NAA level µM	A	J1	A	J1	A	J1	A	J1
0	0	0	0	0	0	0	0	0
0.5	0	0	1	1	0	1	1	0
2.5	0	0	0	0	5	0	1	0
12.5	0	0	0	1	5	0	3	0

3.2 REGENERATION FROM ANTHERS

SUMMARY OF ORDER OF ANTHER CULTURE WORK

Note: For clarity of presentation the work with the model plant is presented first.

	Genotype		Treatments
1.	A	Table 11	Exp. 7 sucrose concentration 5, 10, 15% heat shock period 48, 48, 72 hours at 35°C solid v liquid medium pretreatment at 4°C 2,4D 1mgdm^{-3}
2.	A, B, C	Table 12	Exp. 8 sucrose concentration 10, 15% addition of L serine heat shock period 0, 6, 18, 48 hours at 35°C NAA, 2,4D 1mgdm^{-3} each

3.	A, B, C	Table 13	Exp. 9 sucrose concentration 5, 10% agar concentration 0.7, 0.35% heat shock period 6, 18 hours NAA 2,4D, KIN 1mgdm^{-3} each
4.	A, B, C	Table 14	Exp. 10 sucrose concentration 5, 10% agar concentration 0.7, 0.35% NAA 0.5mgdm^{-3}, BA 0.1mgdm^{-3}
5.	Oilseed rape var Duplo	Table 7	Exp. 5 solid v. liquid medium
6.	Oilseed rape var Duplo	Table 8, 9, 10	Exp. 6 agar v. Gelrite
7.	A, C	Table 15	Exp. 11 agar v. Gelrite heat shock period 6, 18 hours 2,4D, NAA 1mgdm^{-3} each NAA 0.5mgdm^{-3}, BA 0.05mgdm^{-3}

8. J1-J11 Table 16 Exp. 12 Gelrite
sucrose concentration 2 or 10%
2,4D, NAA 1mgdm^{-3} each
NAA 0.5mgdm^{-3},
BA 0.05mgdm^{-3}

Exp. 5. Testing of procedures using a model plant (*Brassica napus* ssp. *oleifera* var Duplo)

The intention was to use a plant related to watercress in which anther culture is an established technique. This was to test the techniques developed for handling watercress anthers.

The seed of a spring cultivar of oilseed rape *Brassica* napus ssp. *oleifera* var. Duplo was kindly supplied by Dr J.M. Dunwell of John Innes Institute, Norwich, and methods adopted were those used successfully for this variety. (Dunwell & Cornish 1985, Dunwell *et al* 1983)

Seeds were sown onto trays of Levington universal compost and placed under mist. After one week the seedlings were potted individually into 10cm pots. Plants were then moved to a Saxcil growth cabinet at 15°C and 20 hours daylength. First visible flower buds appeared after one month.

Two media were used both of which had been successfully used in liquid form with 'Duplo'

1. 2,4D medium - half strength MS with 2,4D 1mgdm^{-3}, NAA 1mgdm^{-3}, 10% sucrose. (Dunwell & Cornish 1985)

2. BA medium - half strength MS with NAA 0.5mgdm^{-3}, BA 0.05mgdm^{-3}, 8% sucrose. (Dunwell *et al* 1983)

Both media were used as liquid and solidified with 0.7% agar (w/v). Gelrite (Kelco) at 0.3% (w/v) was also used on a few plates as an alternative solidifying agent.

Sterilization of anthers was carried out as described earlier for watercress (section 2.2). Inflorescences were harvested just as the first flower opened. Buds used for culture were chosen to be between 2.0 and 3.5mm long. The bud length was recorded on each plate. Other workers have found this range of bud lengths to correspond approximately to uninucleate microspores within the anther (Thurling & Chay 1984). Dissection was carried out under aseptic conditions in a laminar flow cabinet under an Olympus dissecting microscope at a magnification of x40, using sharp sterile scalpels (blade Swann Morton No. 11). Buds were held on sterile distilled water to prevent dehydration until dissection was carried out. Six anthers from a bud were placed on a single 50mm plate which was sealed with "Parafilm".

All plates were incubated initially for 48 hours at 35°C and then moved to 25°C in the dark. All plates were scored for embryo development at 42 days.

A number of embryos were transferred to a regeneration medium which was half strength MS, plus 2.5μM BA and 2% sucrose, as transfer to low sucrose medium is necessary for further development of embryos. Embryos were grown on 50mm plates at 25°C and 16 hours photoperiod for 6 weeks and then subcultured to half strength MS with 2% sucrose but no PGRs, in 100ml Sterilin jars and cultured in the same conditions. Subculturing was repeated every six weeks until the plantlets appeared normal. Plantlets then had the agar carefully removed from their roots and were weaned as described in section 2.8.

Results

The results are shown in table 7. It can be seen that embryos were successfully produced from anthers of *B. napus* ssp *oleifera* var Duplo. It should be noted that five of the embryogenic buds were 2mm long, two were 2.5mm in length and one was 3.5mm in length.

Embryos initially developed into green thalloid structures. These started to produce normal looking shoots after subculture to PGR-free medium. Development of a root system was very slow and it was

not clear that there were vascular connections between shoot and roots as there was usually a substantial development of callus between shoot and roots. After 5 months the plantlets were weaned successfully with 19 out of 20 plantlets surviving. 10 of these plants were grown on to flowering of which 6 produced small sterile flowers and failed to set seed, while 4 produced normal looking flowers and set seed normally.

TABLE 7 no. of induced buds, induced anthers and embryos produced in test of procedure with oilseed rape var. Duplo.

Medium	Total No, Buds	Total No, Anthers	No,Induced Buds	% Induced Buds	No, Induced Anthers	% Induced Anthers	No,Embryos	No, Embryos per Induced Anther
BA liquid	27	162	0	0	0	0	0	0
BA agar	35	210	3	1,43	9	4,29	31	3,44
BA Gelrite	2	12	0	0	0	0	0	0
2,4D liquid	26	156	0	0	0	0	0	0
2,4D agar	34	204	4	1,96	7	3,43	25	3,57
2,4D Gelrite	2	12	1	50,0	5	41,67	37	7,40
TOTAL	126	756	8	6,35	21	2,77	93	4,43

Exp. 6. An investigation into the effect of gelling agent on anther culture in *Brassica napus* ssp *oleifera* var Duplo

Seeds of *Brassica napus* ssp *oleifera* var Duplo, were germinated and grown as described in the previous section (Exp. 5). Flower buds were harvested when the first flower on the infloresence had opened (Dunwell & Cornish 1985). All flower buds used were between 2mm and 3.5mm. The buds were sterilized as in section 2.2 and dissected as described in the previous experiment.

The basal medium consisted of half strength MS (Murashige & Skoog 1962) with 10% sucrose and the PGRs 2,4D at 1mgdm^{-3} and NAA at 1mgdm^{-3} (Dunwell & Cornish 1985). The gelling agents tested were Gelrite (Kelco) at 1, 1.5, 2.0 and 4.0 gdm^{-3} and Lab M code "MC2" agar at 4.0, 5.5, 7.0 and 8.5 gdm^{-3}. There were 72 anthers for each treatment. Anthers were incubated at 35°C for 48 hours in the dark and then at 25°C in the dark for 6 weeks. Both embryo production and anther-derived callus production were scored.

Results

It can be seen from Table 8 that the gelling treatment does have an effect on anther culture of oilseed rape. 1gdm^{-3} of Gelrite in the medium gave more productive anthers and a higher value for the average number of

embryos per productive anther than any other treatment. χ^2 factorial analysis of the number of anthers producing embryos (Table 9) indicated that the level of either agar or Gelrite in the medium was significant at the P = 1% level. The type of gelling agent was also significant at the P = 5% level. χ^2 factorial analysis was also carried out on the number of anthers producing anther-derived callus (Table 10) and this indicated that the type of gelling agent was significant at the P = 1% level while the level of gelling agent was significant at the P = 5% level.

Exp. 5. Testing of procedures using a model plant.

3 embryo formation from an anther of oilseed rape var Duplo on the 2,4D medium containing Gelrite x 4.4

TABLE 8. Effect of gelling agent on anther culture of oilseed rape.

Treatment gdm^{-3}		Total no. of anthers	No. of embryos produced	No. of anthers producing embryos	No. of buds producing embryos	No. of anthers producing callus	No. of buds producing callus
GELRITE	1,0	72	22	7	3	6	3
	1,5	72	1	1	1	3	2
	2,0	72	1	1	1	0	0
	4,0	72	1	1	1	1	1
AGAR	4,0	72	2	2	2	0	0
	5,5	72	0	0	0	1	1
	7,0	72	0	0	0	0	0
	8,5	72	2	1	1	0	0

TABLE 9. Effect of gelling agent χ^2 factorial analysis on the number of anthers of oilseed rape producing embryos.

source	χ^2	df	significance
type of gelling agent	3.857	1	*
level of gelling agent	14.08	3	***
residual variance	4.73	3	n.s.
whole experiment	22.68	7	***

TABLE 10. Effect of gelling agent χ^2 factorial analysis on the number of anthers of oilseed rape producing callus

source	χ^2	df	significance
type of gelling agent	7.5	1	***
level of gelling agent	8.43	3	*
residual variance	7.7	3	n.s
whole experiment	23.63	7	***

Exp. 7. Anther culture of watercress

The experiments presented in tables 11-14 were carried out before the work with oilseed rape. The treatments presented in tables 15 and 16 were carried out in the light of the beneficial effect of the use of Gelrite for anther culture of oilseed rape, but for clarity of presentation the model plant work is presented first.

The optimum stage of pollen development is of vital importance in the success of anther culture. Therefore an attempt was made to correlate easily measurable external morphological factors to pollen development stage. The optimum stage varies between species but in *Brassica ssp* early uninucleate pollen seems to be most responsive.

Buds were removed from the inflorescence. Bud length was measured using an eyepiece graticule. It was then carefully dissected, and anther length and petal length were measured. The anther was then squashed in acetocarmine and examined microscopically to determine stage of pollen development.

Watercress buds of 0.8mm to 1.2mm in length were harvested when the first flower in the infloresence had opened. They were sterilized as described in section 2.2 and then dissected under aseptic conditions under an Olympus dissecting microscope at a magnification of

x40. All 6 anthers from a bud were placed on the same plate which was sealed with Parafilm.

Six experiments were set up to test a range of media and other influences on anther culture of watercress.

Incubation was carried out in the dark at 25°C with thermal shock treatments at 35°C immediately after excision as indicated in tables 11-16. The compositions of the various media tested, the genotype used and the numbers of anthers tested are also indicated in tables 11-16. Unless otherwise indicated media were solidified with Lab M "MC2" agar at 7gdm^{-3} and were made up as described in section 2.5. After 6 weeks cultures were scored for embryo production or anther derived callus formation. Anthers were also squashed in both acetocarmine and Snow's (1963) alcoholic hydrochloric carmine to detect any dividing microspores.

The regeneration medium was half MS, 2% sucrose (w/v) with 2.5μM BA, and culture was at 25°C with a 16 hour photoperiod.

Results

From the measurements taken the petal length:anther length ratio was calculated. The only pollen stages that could be distinguished are listed below, as a

thick exine forms very early in watercress. The stages were arbitrarily assigned the numbers given, for analysis

mother cells	1
tetrads	2
pollen	3.

Analysis of variance was carried out both for petal:anther ratio against pollen stage, and for bud length against pollen stage (Figs 7 & 8).

The statistical tests showed that both bud length and petal:anther ratio were significantly correlated to stage of pollen development at the P = 5% level. It must be noted that these data are somewhat biased in that only buds of a middle size were selected, i.e. 0.8-1.2mm, smaller and larger buds being at mother cell and pollen stages respectively.

Six weeks after the start of culture, watercress anthers were observed and stained with acetocarmine (Evans *et al* 1981a) or Snow's alcoholic hydrochloric carmine.

No embryo production was seen in any cultures, and neither staining with acetocarmine or Snow's (1963) alcoholic hydrochloric carmine revealed any dividing microspores. Anthers either became brownish green and

FIG. 7. Analysis of variance of petal length:anther length ratio against pollen stage

due to	df	ss	ms=ss/df	f-ratio
factor	2	0.2970	0.1485	11.13 ·
error	29	0.3870	0.0133	
total	31	0.6840		

level	n	mean	st. dev.
1	6	0.508	0.069
2	10	0.673 ·	0.096
3	16	0.767	0.137

level
1 = pollen mother cells
2 = tetrads
3 = pollen

pooled st. dev. = 0.116

individual 95 percent c. i. for level means
(based on pooled standard deviation)

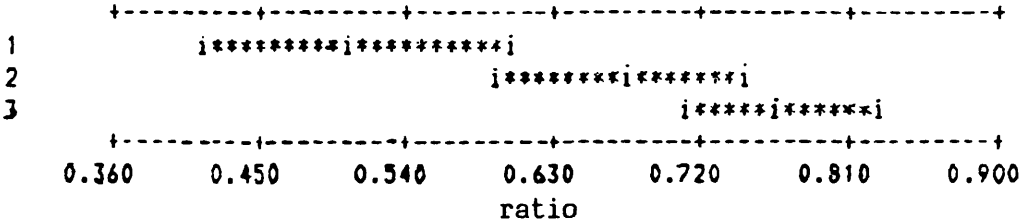


FIG. 8. Analysis of variance of bud length against pollen stage

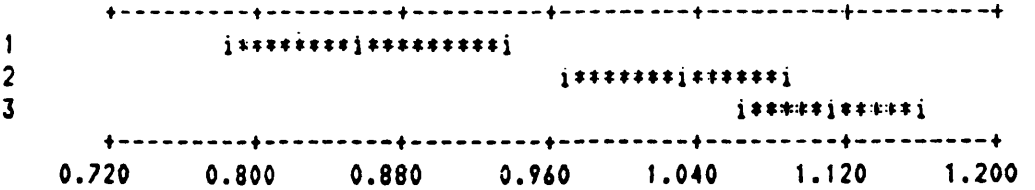
due to	df	ss	ms=ss/df	f-ratio
factor	2	0.28378	0.14189	16.90
error	29	0.24351	0.00840	
total	31	0.52729		

level	n	mean	st. dev.
1	6	0.8567	0.0350
2	10	1.0300	0.0675
3	16	1.1112	0.1144

level
1 = pollen mother cells
2 = tetrads
3 = pollen

pooled st. dev. = 0.0916

individual 95 percent c. i. for level means
(based on pooled standard deviation)



expanded slightly or became translucent and did not develop. Anthers on liquid media rapidly sank, they probably became anaerobic and died as no callus development was seen. Some of the brownish anthers produced callus from the filament which showed occasional root formation and eventually turned black. Thermal shock treatments of 72 hours caused most of the anthers to become translucent and as no filament callus developed on these anthers it was concluded that this treatment was lethal. The smaller anthers also became translucent at 48 hours which indicated that they were more vulnerable to the heat shock than larger anthers. The shorter thermal shock period of 18 hours was therefore chosen as a maximum in later experiments. It was notable that not all anthers on a plate responded to culture in the same manner.

Callus which was apparently anther-derived appeared on one anther of J1 on the BA medium with 10% sucrose (Table 16) (plate 4a). This enlarged but became brown and stopped developing on transfer to the regeneration medium. A tiny rounded pale structure appeared on J1 on the 2,4D (Table 16) (plate 4b) medium with 10% sucrose and on J7 on the same medium (Table 16) but these could not be stimulated into further development and their nature i.e. callus, early embryoid or somatic tissue proliferation remained unclear.

Exp. 12. Effect of genotype and Gelrite on anther culture of watercress

4a possible anther derived callus from anther of J1 on the BA medium with 10% sucrose x 2.8

4b possible microspore derived structure from anther of J1 on the 2,4D medium with 10% sucrose x 11

TABLE 11. Exp. 7. Effect of sucrose concentration, gelling agent and incubation period - no. of anthers per treatment.

Clone A Medium ½ MS 1mgdm^{-3} 2,4D (Dunwell pers.comm.)

% sucrose	gelling agent	pretreatment at 4°C	incubation period at 35°C (hours)		
			18h	48h	72h
5	agar	36	60	156	36
10	agar	36	60	156	36
15	agar	-	60	84	-
5	none	36	60	144	36
10	none	36	60	138	36
15	none	36	-	54	36
Total no. anthers		180	300	678	180

TABLE 12. Exp. 8. Effect of genotype and incubation period - no. of anthers per treatment.

Medium ½ MS 1mgdm^{-3} 2,4D 1mgdm^{-3} NAA 0,7% agar, L-serine at 100mgdm^{-3}

ref. (Keller & Armstrong 1977, 1983)

			incubation period at 35°C (hours)					
			0	6	18	48	6	6
% sucrose	addition	clone	A	A	A	A	B	C
10	L-serine		42	12	48	48	30	30
10	-		36	12	48	48	18	18
15	-		36	6	42	48	18	18
Total no. Anthers			114	30	138	144	66	66

TABLE 13. Exp. 9. Effect of genotype and PGRs - no. of anthers per treatment.

Medium M MS 1mgdm^{-3} of each NAA, 2,4D, KIN (Cheng 1983)

	clone	A		B		C
	incubation time hours	6	18	6	18	18
% sucrose	% agar					
5	0,7	18	36	-	30	36
10	0,7	18	36	72	30	36
10	0,35	18	36	24	30	48
Total no. anthers		54	108	96	90	120

TABLE 14. Exp. 10. Effect of genotype and PGRs - no. of anthers per treatment.

Medium M MS 0,5mgdm^{-3} NAA, 0,1mgdm^{-3} BA (Lichter 1981)

	clone	A		B		C
	incubation time	6	18	6	18	18
% sucrose	% agar					
5	0,7	18	36	-	30	24
10	0,7	18	36	48	30	36
10	0,35	18	36	-	30	36
Total no. anthers		54	108	48	90	96

TABLE 15. Exp. 11. Effect of Gelrite and PGRs

- no. of anthers cultured.

Medium 2,4D = ½ MS 10% sucrose (w/v) 1mgdm^{-3} 24D 1mgdm^{-3} NAA
(Dunwell & Cornish 1985)

BA = ½ MS 8% sucrose (w/v) 0,5mgdm^{-3} NAA 0,05mgdm^{-3} BA
(Dunwell *et al* 1983)

		incubation time hours		
		6	18	
medium	clone gelling agent	A	A	C
BA	agar	48	138	24
BA	Gelrite	-	84	36
2,4D	agar	48	138	24
2,4D	Gelrite	-	102	36
Total no. anthers		96	462	120

TABLE 16. Exp. 12. Effect of genotype and Gelrite

- no. of anthers cultured.

Medium 2,4D = ½ MS 1mgdm^{-3} 2,4D 1mgdm^{-3} NAA 0,2% Gelrite
(Dunwell & Cornish 1985)

BA = ½ MS 0,5mgdm^{-3} NAA, 0,05mgdm^{-3} BA 0,2% Gelrite
(Dunwell *et al* 1983)

clone											
medium	% sucrose	J1	J2	J3	J4	J5	J6	J7	J8	J9	J10	J11
BA	2	96	36	48	36	36	36	96	36	36	36	36
BA	10	96	36	96	36	36	96	102	36	36	36	36
2,4D	2	96	36	96	36	36	96	96	36	36	36	36
2,4D	10	150	150	102	144	144	102	102	72	96	36	36
Total no.anthers		438	258	342	252	252	330	396	180	204	144	144

3.3 EXP. 13. REGENERATION FROM POLLEN

Flower buds of various strains of watercress (shown in Table 17) were harvested when the first flower on the inflorescence had opened. They were surface sterilised as in section 2.2 and then were macerated in Gamborg's B_5 medium (1968) (with 13% sucrose w/v) with a PTFE homogenizer. All equipment was sterilised by autoclaving and pollen isolation was carried out under aseptic conditions. All media were kept cool in an incubator (about 12°C) as far as possible, as Swanson *et al* (1987) found this to be the most effective temperature for *Brassica napus*.

The slurry was passed through a polyester mesh of 30μM and collected in a petri dish. Measurement of the diameter of pollen grains of watercress under a microscope using an eyepiece graticule showed that the maximum size was 23μm and a preliminary test had shown that they passed a mesh of this size. The equipment was rinsed through with B_5 medium and then the liquid was pipetted into centrifuge tubes. These were balanced and then centrifuged at 350g for 10 minutes. The supernatant was poured off and the pellet was resuspended in cool B_5. This was repeated twice, and then the pellet was resuspended in pollen culture medium. This medium was NN (Nitsch & Nitsch 1969) as modified by Lichter (1981, 1982) except without potato

extract i.e. with 13% sucrose (w/v), 30mgdm^{-3} glutathione, 800mgdm^{-3} L-glutamine and 10mgdm^{-3} L-serine. Both PGR-free NN (as Swanson et al 1987) and NN containing 0.05mgdm^{-3} BA and 1mgdm^{-3} NAA (as Chuong & Beversdorf 1985) were tested. The pollen suspension was incubated overnight in the dark at 30°C. It was recentrifuged and the supernatant poured off and then resuspended in fresh pollen culture medium. The pollen was counted using an improved Neubauer counting chamber and was plated at approximately 75,000 pollen grains per ml. 0.6ml of solution was pipetted into each 30mm petri dish, a volume calculated to give a liquid layer of the same depth as used by Swanson *et al* (1987) & Nitsch (1981) who points out that a thin layer is essential to ensure good aeration. The cultures were then sealed with Parafilm, and two dishes, one with PGRs and one without, were placed in a 90mm petri dish.

The pollen was incubated at 30°C for 14 days in the dark. It was then diluted with two volumes of medium and cultured at 25°C in the dark and 50 r.p.m. Scoring for embryo formation was carried out after a further 14 days.

Results

The experimental layout is shown in Table 17.

No embryo formation was seen in any of the cultures, and microscopic examination of cultures, both unstained and stained with Snow's (1963) alcoholic hydrochloric carmine, did not reveal any dividing microspores.

TABLE 17 Regeneration from pollen - experimental layout

Strain of watercress	Clonal line	No. Plates	
		Without PGR	With PGR
USA	1	16	17
USA	2	4	4
AUS	1	10	10
AUS	2	6	6
NZ	1	12	12
NZ	2	28	26

3.4 EXP. 14. REGENERATION FROM OVULES

Watercress flower buds in the size range 1-2mm were harvested. They were sterilized as in section 2.2 and were dissected under aseptic conditions. Care was taken not to rupture anthers which might have led to contamination of ovules with pollen. The pistil was carefully slit lengthways with a sharp scalpel (blade Swann Morton No. 11) and the ovules scraped out. These were spread onto the surface of the medium, with the ovules from one flower bud per petri dish.

The media were those used for anther culture of oilseed rape (Exp. 5) solidified with 7gdm^{-3} Lab M "MC2" agar or 2gdm^{-3} Gelrite (Kelco)

i.e. BA medium - half strength MS with 0.5mgdm^{-3} NAA, 0.05 mgdm^{-3} BA + 8% sucrose (Dunwell *et al* 1983)

2,4D medium - half strength MS with 1mgdm^{-3} 2,4D, 1mgdm^{-3} NAA + 10% sucrose (Dunwell & Cornish 1985).

Half the plates were given a heat shock at 35°C for 3 hours before culture at 25°C in the dark, while the rest were incubated immediately at 25°C. Six replicates of each treatment were set up.

Any calli or embryos formed were transferred to a regeneration medium containing half strength MS, 2%

sucrose and 2.5μM BA (as used in Exp. 5) and incubated at 25°C and in a 16 hour photoperiod.

Results

The experimental layout and observations are shown in Table 18.

Callus formation occurred from one ovule on the BA medium with agar and from one ovule on the BA medium with Gelrite, both cultured at 25°C. After transfer to the regeneration medium the first callus did not develop further and did not turn green. Part of the second callus became green and a root like structure formed, but the callus died after about 4 weeks with no further development. No callus formed in cultures which had had a heat shock.

TABLE 18 regeneration from ovules - experimental layout and observations. n = 6

Medium	Gelling agent	Temp °C regime	Observation
BA	agar	35	
BA	agar	25	1 callus on one ovule
BA	Gelrite	35	
BA	Gelrite	25	1 callus on one ovule
2,4D	agar	35	
2,4D	agar	25	
2,4D	Gelrite	35	
2,4D	Gelrite	25	

BA medium = half strength MS, 0.5mgdm^{-3} NAA, 0.05mgdm^{-3} BA, 8% sucrose
2,4D medium = half strength MS, 1mgdm^{-3} 2,4D, 1mgdm^{-3} NAA, 10% sucrose
agar = 0.7% w/v
Gelrite = 0.2% w/v

3.5 EXP. 15. LONG TERM STORAGE

Nodal cultures of each clone tested were established as described earlier in sections 2.4 and 2.6.

After four weeks, shoot tips 10-15mm in length were produced which were used in all long term storage experiments. Three shoot tips were placed on 25ml of medium in 60ml "Sterilin" screw-topped plastic jars. The control medium was MS, with 2% sucrose and 0.7% (w/v) agar. The experiments also contained the following additional treatments which were chosen on the basis of their successful use in other species, as discussed in section 1.3.

(1) mannitol at 1%, 3% and 6% (w/v),
(2) ABA at 5, 10 and 20mgdm^{-3},
(3) paclobutrazol at 0.2, 2, 5, 25 ppm,
(4) sucrose at 4%, 8%, 12% w/v,
(5) low temperature of 10°C.

30 jars were set up per treatment and maintained in 16 hours light + 8h dark and 25°C unless otherwise stated. Experiments were carried out with the clonal line of English Dark Green watercress labelled A and the clonal line of Japanese watercress labelled J1. Genotypes may differ in their response to storage treatments, but

limited space prevented tests on more than two genotypes.

Survival of cultures was assessed by the ability to produce new growth when shoot tips were transferred to fresh PGR-free MS medium. Fifteen jars were assessed at six months and the remaining fifteen jars were assessed at twelve months. The ability of shoot tips to survive the weaning process was also tested. After two to four weeks on fresh MS medium shoot tips were weaned by transferring onto a 2:1 (w/v) mixture of Levington universal compost and Silvaperl perlite in small trays. They were treated as described in section 2.8.

Results

In these experiments only a clear cut improvement in survival would be of value for a commercial long term storage programme. Statistical analysis of results showing little or no imporovement was therefore not justified.

Watercress shoot tips survived for a surprisingly long period in tissue culture though by 6 months there was some dead material present, as well as the living material. At 12 months 19% of clone A control shoots were surviving while 29% of J1 control shoots were still alive.

Table 19 shows the effect of inclusion of mannitol in the medium. As this sugar is metabolically inactive, the only effect is to increase the osmotic pressure of the medium which is believed to have a general metabolic effect on the explant growth rate (Henshaw et al 1980). Inclusion of 1% (w/v) mannitol in the medium gave a slight improvement in survival in J1 and slight decrease in survival in A. However the shoots were smaller; with shortened internodes and less dead tissue. In both A and J1 survival of weaning was slightly improved by the 1% mannitol treatment compared to the control. At the higher mannitol concentrations, 3% and 6%, the explants were stunted and a considerable amount of vitrification was seen with poor survival rates at 6 months.

Table 20 shows the effect of ABA in the medium. For clone A 5mgdm^{-3} ABA gave survival similar to the control at 6 months over the control and there was a marked improvement in survival of weaning, though survival at 12 months was very low. Clone J1 responded quite differently with only 9% survival at 6 months on 5mgdm^{-3} ABA. The higher concentrations of ABA gave lower survival rates than the control for both clones. Explants on ABA had larger leaves than the control, but these were pale green and there was considerable formation of hairy axillary roots from the nodes. There was a notable difference in response within a clone to ABA which may be due to slight differences in

age and therefore physiological status in the initial explant.

Table 21 shows the effect of inclusion of the gibberellin biosynthesis inhibitor, paclobutrazol in the medium. This acts to limit internode elongation, and produced a range of degrees of stunting in both clones. The leaves were darker in colour than the control and at 0.2, 1 and 5 ppm were increased in size. Survival rates were lower than the control, particularly at the higher concentrations.

Table 22 shows the effect of sucrose at a range of concentrations on shoot survival. All treatments gave lower survival rates than the control and at higher concentrations the survival rate was extremely low. There was a noticeable difference in response between the two clones A and J1. During the first two months of the experiment shoots were larger, with big leaves and a dark colour but died at about 3 months.

Table 23 shows the effect of low temperature on survival of watercress shoot tip cultures. There was a very high survival rate of 98% for both A and J1 at 6 months which was a marked improvement over the controls. Survival of weaning was also better for shoots maintained at low temperatures compared to the control. Survival at 12 months was 80% for clone A and 73% for J1 which was considerably better than the

controls (19% and 29% respectively). There was also a high rate of survival of weaning in both clones from low temperature treatments. The improvement in survival was sufficiently marked that statistical analysis was unnecessary.

TABLE 19

EFFECT OF MANNITOL ON PERCENTAGE SURVIVAL OF WATERCRESS SHOOT TIP CULTURES
n = 45

TREATMENT % (w/v)	CLONAL LINE	% SURVIVAL AFTER 6 MONTHS	% SURVIVAL AFTER WEANING	% SURVIVAL AFTER 12 MONTHS	% SURVIVAL AFTER WEANING
CONTROL	A	74	47	19	15
	J1	64	61	29	27
1	A	66	54	-	-
	J1	71	64	-	-
3	A	7	4	0	0
	J1	0	-	-	-
6	A	7	2	0	0
	J1	0	-	-	-

- indicates not tested

TABLE 20

EFFECT OF ABSCISIC ACID ON PERCENTAGE SURVIVAL OF WATERCRESS SHOOT TIP CULTURES
n = 45

TREATMENT $mgdm^{-3}$	CLONAL LINE	% SURVIVAL AFTER 6 MONTHS	% SURVIVAL AFTER WEANING	% SURVIVAL AFTER 12 MONTHS	% SURVIVAL AFTER WEANING
CONTROL	A	74	47	19	15
	J1	64	61	29	27
5	A	71	62	2	2
	J1	11	9	2	2
10	A	42	40	0	0
	J1	27	27	2	2
20	A	20	18	0	0
	J1	33	33	4	4

TABLE 21

EFFECT OF PACLOBUTRAZOL (AS "BONZI") ON PERCENTAGE SURVIVAL OF WATERCRESS SHOOT TIP CULTURES
n = 45

TREATMENT ppm	CLONAL LINE	% SURVIVAL AFTER 6 MONTHS	% SURVIVAL AFTER WEANING	% SURVIVAL AFTER 12 MONTHS	% SURVIVAL AFTER WEANING
CONTROL	A	74	47	19	15
	J1	64	61	29	27
0.2	A	31	31	0	0
	J1	20	20	0	0
1	A	11	0	0	0
	J1	22	22	0	0
5	A	0	0	0	0
	J1	2	2	0	0
25	A	2	0	0	0
	J1	4	4	0	0

TABLE 22

EFFECT OF SUCROSE ON PERCENTAGE SURVIVAL OF WATERCRESS SHOOT TIP CULTURES n = 45

TREATMENT % (w/v)	CLONAL LINE	% SURVIVAL AFTER 6 MONTHS	% SURVIVAL AFTER WEANING	% SURVIVAL AFTER 12 MONTHS	% SURVIVAL AFTER WEANING
CONTROL	A	74	47	19	15
	J1	64	61	29	27
4	A	53	51	0	0
	J1	40	40	0	0
8	A	4	4	0	0
	J1	0	0	0	0
12	A	7	7	0	0
	J1	0	0	0	0

TABLE 23

EFFECT OF LOW TEMPERATURE ON PERCENTAGE SURVIVAL OF WATERCRESS SHOOT TIP CULTURES
n = 45

TREATMENT	CLONAL LINE	% SURVIVAL AFTER 6 MONTHS	% SURVIVAL AFTER WEANING	% SURVIVAL AFTER 12 MONTHS	% SURVIVAL AFTER WEANING
CONTROL	A	74	47	19	15
	J1	64	61	29	27
10°C	A	98	95	80	78
	J1	98	98	73	69

Exp. 15. Effect of low temperature on survival of watercress shoot tips in culture.

5a J1 on the left at 10°C compared to control on the right

5b A on the left at 10°C compared to control on the right

3.6 ASPECTS OF THE BIOLOGY OF WATERCRESS *IN VIVO*

Exp. 16. Investigation into the self-compatibility of watercress

To confirm that watercress is self-compatible 30 clonal plants of line A were placed in a Saxcil growth cabinet. They were allowed to flower and set seed, which was harvested just before dehiscence. To investigate whether there was any loss of fecundity associated with self-pollination, a number of pods were harvested at the same developmental stage from plants grown in the field at Hampshire. Seed counts per pod were made for 30 pods of each and the results analysed.

Results

Numbers of seeds per pod for selfed pods and for field pods are shown in Table 24.

Both Students t test (t = 2.67) and a variance ratio test (F = 1.4) showed that there was no significant difference between numbers of seeds per pod (Table 25).

TABLE 24 - no. seeds per pod

pod no.	field pods	selfed A pods
1	30	35
2	32	17
3	23	40
4	21	26
5	37	24
6	45	30
7	44	26
8	26	35
9	28	28
10	33	25
11	37	30
12	31	35
13	42	21
14	30	21
15	32	32
16	29	43
17	30	29
18	43	31
19	21	25
20	19	32
21	25	26
22	34	20
23	29	26
24	33	22
25	39	28
26	30	22
27	40	28
28	32	30
29	36	29
30	41	22

TABLE 25. Analysis of no. of seeds per pod for field pods and selfed pods.

	Field pods	Self pods
n	30	30
ϵx	972	832
ϵx^2	32916	24420
$\bar{x}$	32.4	27.9
γ_{n-1}	7.005	5.906
s^2	49.07	34.88
df	29	29

Exp. 17. Investigation into self-pollination in watercress

Eight plants of Line A were placed in isolation from each other and from any other sources of watercress pollen. All inflorescences except the apical one were removed while still closed and all flower buds except one were carefully removed. The remaining single flower bud was allowed to open and develop.

Microscopic examination under a x40 Olympus dissecting microscope was carried out on flower buds from other plants.

Results

Three plants developed seed pods which aborted at an early developmental stage, while 5 plants set seed as normal giving seed counts per pod of 17, 40, 26, 35 and 24 respectively. Microscopic examination indicated that anther dehiscence can occur before the flower is open sufficiently for wind or insect vectors, and there is some pollen transfer to the stigma at this stage.

Exp. 18. Comparison of the germination rates of selfed seed versus field seed

Seed material was selfed seed of Line A and seed from the field at Fobdown Farm. Ten seeds of each were

placed on damp filter paper in 90mm petri dishes which were sealed with Parafilm. Five replicates of each were set up. Incubation was carried out in a Conviron growth cabinet at 20°C with 20 hours light plus 4 hours dark. Germination was scored at 14 days.

Results

Table 26 shows that there is no great difference between numbers of seeds germinating and statistical analysis by χ^2 confirmed that there was no significant difference between the number of seeds germinating.

TABLE 26.

Comparison of number of seeds germinating for selfed seed versus field seed n = 10

Replicate	No. germinated	
	Selfed A	Field
1	8	9
2	9	8
3	7	8
4	8	10
5	8	8
Total	40	43

Exp. 19. Effect of clonal seed parents on the uniformity of a watercress crop

A previous worker (unpublished) had bulked up two visually distinct watercress plants labelled x and y using tissue culture techniques. These were then weaned and planted out in experimental beds at Fobdown Farm. Seed from both lines was collected separately and stored desiccated at -20°C.

Seed of x, y and a control batch of seed from the standard commercial seed line, were sown in the commercial propagation system and were planted out into three adjacent crop beds. One week before harvest they were assessed.

A stratified random sampling method was used. Each bed was divided up into 4 numbered sections, ignoring a 30cm margin around the concrete bed walls, where turbulent water flow prevents plant establishment. One 10cm quadrat was taken in each section whose position was determined by random numbers, though if the quadrat was located in a bare patch it was placed again. This was justified as failure of plants to establish in these patches was due to seedlings being washed away not a failure of the plant itself. All plants rooted within the quadrat were cut off close to the gravel with sharp scissors. Each sample was placed in a

labelled plastic bag and stored at 4°C until it could be measured.

Measurements taken were

(1) Total number of stems
(2) Number of marketable stems (defined as those stems over 80cm long and 2mm in diameter).

The marketable stems were further measured for

(3) plant height,
(4) length of the second and third internodes,
(5) length and width of the leaves at the bottom of the second internode and top of the third internode.

The ratio of length to width was used to give an indication of leaf shape.

The measurements were then analysed.

Results

Both plants from x and y (plate 6a) gave crops that appeared more even in terms of leaf shape and establishment than the control.

Tables 1-9 in Appendix C give the measurements taken from the plants.

Fig. 9 shows the analysis of variance of plant height in each bed. It can be seen that plants from x are slightly taller than the control, but as the 95% confidence intervals overlap this is not a significant difference. However plants from y are significantly taller than the control.

Fig. 10 shows analysis of variance according to position in each bed. This shows that there is an effect of position with plants from section 1 being the shortest and from section 2 being the tallest.

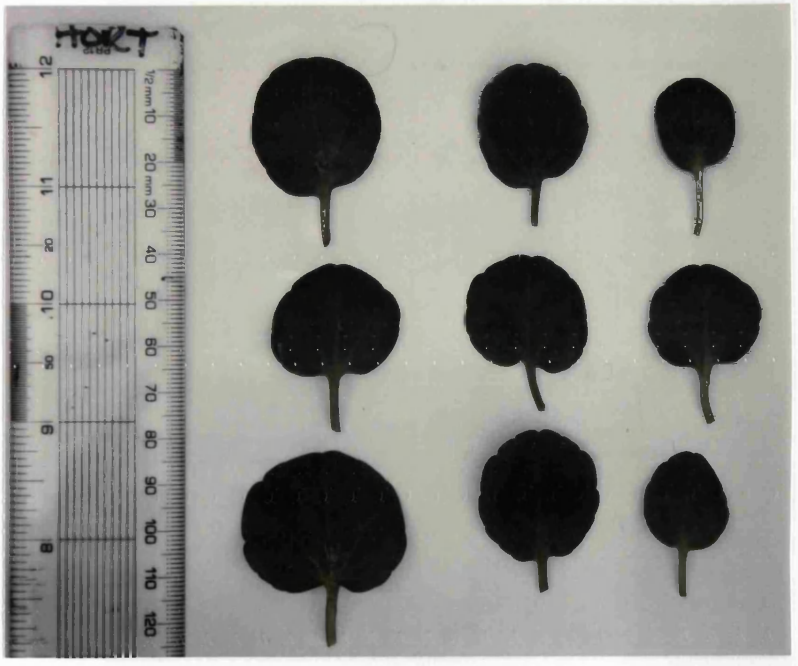
Fig. 11 shows the analysis of variance of all the internode lengths. Plants of x have slightly longer internodes than the control plants, but this is not a significant difference. However plants of y show a highly significant increase in internode length.

Fig. 12 shows the analysis of variance of leaf shape ratios. This shows that plants from both x and y have significantly different shapes from the control. Leaves of x are wider than long and leaves of y are longer than wide. Plate 6b shows the differences in leaf shape.

Fig. 13 shows the analysis of the numbers of marketable stems, the number of unmarketable stems and the total number of stems. The most important of these from a commercial aspect is the number of marketable stems.

Exp. 19. Effect of clonal seed parents on a watercress crop

6a plants from y on the left and from x on the right



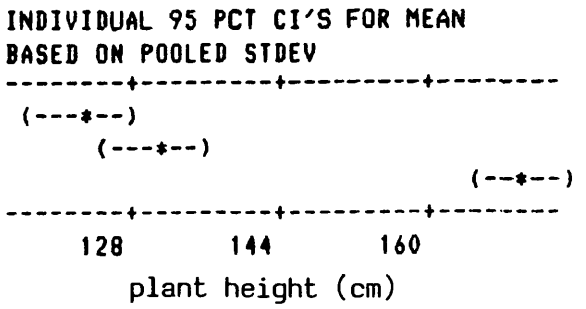
6b variation in leaf shape in y (top row) x (2nd row control (3rd row)

FIG. 9 . Analysis of variance of plant height.

SOURCE	DF	SS	MS	F
FACTOR	2	93924	46962	93.36
ERROR	198	99602	503	
TOTAL	200	193526		

LEVEL	N	MEAN	STDEV
C139	55	122.40	20.02
C140	59	130.83	23.26
C141	87	169.92	23.27

POOLED STDEV = 22.43



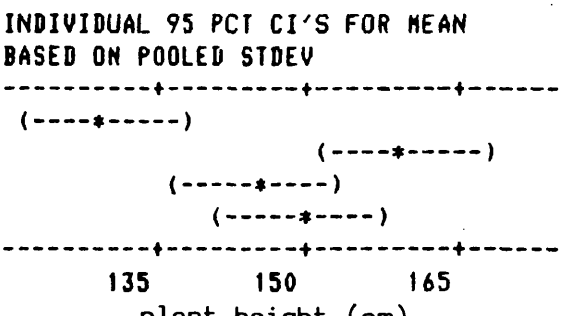
C139 = plant heights of control
C140 = plant heights of x
C141 = plant heights of y

FIG. 10. Analysis of variance of plant height according to position in bed.

SOURCE	DF	SS	MS	F
FACTOR	3	23458	7819	9.06
ERROR	197	170068	863	
TOTAL	200	193526		

LEVEL	N	MEAN	STDEV
C142	52	129.40	30.29
C143	47	159.45	34.61
C144	55	145.00	25.69
C145	47	149.70	26.59

POOLED STDEV = 29.38



C142 = quadrat 1 in each bed
C143 = quadrat 2 in each bed
C144 = quadrat 3 in each bed
C145 = quadrat 4 in each bed

FIG. 11. Analysis of variance of internode lengths.
C125 = control internodes C127 = x internodes C128 = y internodes

SOURCE	DF	SS	MS	F
FACTOR	2	8081.0	4040.5	82.84
ERROR	399	19461.6	48.8	
TOTAL	401	27542.5		

LEVEL	N	MEAN	STDEV
C125	110	16.700	6.158
C127	118	18.000	7.092
C128	174	26.368	7.389
POOLED STDEV =		6.984	

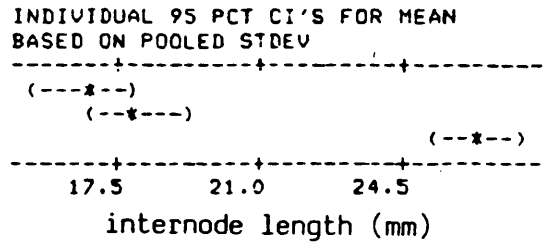


FIG. 12. Analysis of variance of leaf shape ratios.
C122 = control leaves C123 = x leaves C124 = y leaves

SOURCE	DF	SS	MS	F
FACTOR	2	0.55994	0.27997	32.42
ERROR	517	4.46485	0.00864	
TOTAL	519	5.02478		

LEVEL	N	MEAN	STDEV
C122	110	0.9995	0.0802
C123	118	0.9672	0.1004
C124	292	1.0453	0.0942
POOLED STDEV =		0.0929	

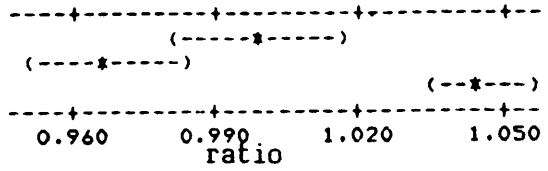


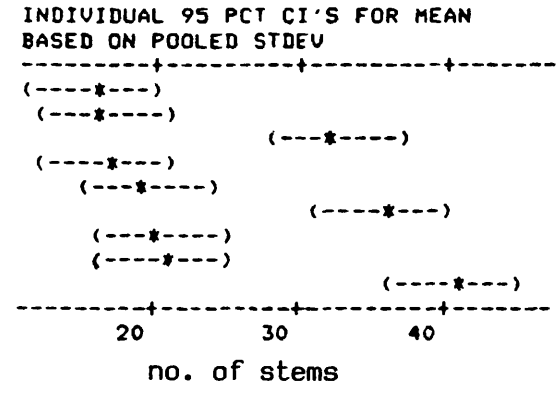
FIG.13. Analysis of Variance of nos. of marketable stems, unmarketable stems and total stems.

conmark	= control marketable stems	xmark	= x marketable stems	ymark	= y marketable stems
conunmark	= control unmarketable stems	xunmark	= x unmarketable stems	yunmark	= y unmarketable stems
contotal	= control total stems	xtotal	= x total stems	ytotal	= y total stems

SOURCE	DF	SS	MS	F
FACTOR	8	11518.1	1439.8	16.98
ERROR	135	11444.4	84.8	
TOTAL	143	22962.5		

LEVEL	N	MEAN	STDEV
conmark	16	15.812	5.902
conunmark	16	16.437	8.862
contotal	16	32.250	13.264
xmark	16	16.562	4.442
xunmark	16	19.250	8.645
xtotal	16	35.813	11.617
ymark	16	20.250	4.524
yunmark	16	20.875	9.010
ytotal	16	40.938	11.941

POOLED STDEV = 9.207



Plants of x show no improvement over the control, while plants of y show a slight improvement which is not significant.

Exp. 20. Introduction of Regenerated Shoots into the Commercial Propagation System

Ten shoots of clone A regenerated from callus (section 3.1) were selected at random. These were numbered 1-10 and were regenerated from the following treatments

1. 12.5μM Dropp 250mgdm^{-3}CH
2. 12.5μM BA 12.5μM NAA
3. 2.5μM Dropp light
4. 12.5μM Dropp 2.5μM 2,4D
5. 12.5μM Dropp transferred to PGR-free medium
6. 0.5μM Dropp with 2.5μM NAA throughout
7. 12.5μM Dropp 2.5μM 2,4D
8. 2.5μM BA 12.5μM NAA
9. 12.5μM Dropp 500mgdm^{-3}CH
10. 12.5μM Dropp "MC29" agar

These were multiplied by shoot tip culture onto PGR-free MS medium in 50mm petri dishes and cultured as described in section 2.6. This was repeated after four weeks. After a further four weeks the material was transported to Hampshire Watercress Ltd.

Shoot tip cuttings 10-20mm in length from each line were transferred to modular trays filled with damp blocking compost. Each tray consisted of 140 modules which were 20 x 20 x 40mm. The cuttings were kept moist by misting and then the trays were transferred to the glasshouse under an overhead mist boom (see section 1.1).

The cuttings were assessed for establishment after two weeks and were then planted out into experimental beds at Fobdown. They were further assessed after four weeks in the experimental beds and the height of 15 plants of each line was measured.

Results

140 cuttings of line 1 to 7, 70 cuttings of line 8 and 47 cuttings of line 9 were transferred. All cuttings of line 10 died during the first four week multiplication. Cuttings of lines 8 and 9 failed to establish in the propagation system. Survival of lines 1 to 7 is shown in table 27.

Table 28 shows the heights of 15 plants of lines 1 to 7 after 4 weeks in a crop bed.

TABLE 27 Survival of weaned cuttings after 14 days.

line	No. surviving	No. dying	Total
1	118	22	140
2	123	17	140
3	130	10	140
4	84	56	140
5	138	2	140
6	132	8	140
7	122	18	140
Total	847	133	980

There was a considerable difference in the appearance of the seven lines of watercress in the bed. The most obvious was in plant height with line 4 being very short and line 6 the tallest. Lines 5 and 7 showed more tillering than other lines. Line 4 had very short internodes, copious rooting at the nodes, and the thickest stems. Line 7 also showed more of the broken leaf shape that usually occurs with flower initiation than any other line, though flower buds were not present. There was also some variation in leaf shape.

χ^2 analysis of numbers of shoots surviving and dying for lines 1 to 7 indicated that there was a significant difference at P = 0.01%.

Analysis of variance was carried out on the plant heights of lines 1 to 7 and Fig. 14 shows that there is a significant difference between various lines.

TABLE 28 Heights of 15 plants of each regenerated line (in cm.)

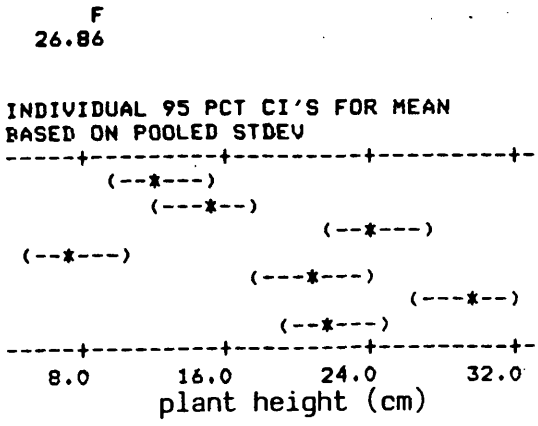
LINE	1	2	3	4	5	6	7
	19	10	30	8	19	30	10
	11	19	18	7	28	23	22
	11	11	26	5	22	18	24
	7	13	31	6	12	37	19
	13	12	18	14	15	37	27
	6	11	33	10	23	21	28
	22	27	23	12	17	38	20
	16	16	20	10	24	28	19
	5	16	16	12	24	22	17
	18	12	26	2	33	22	27
	7	12	32	3	25	30	30
	16	18	24	6	26	34	19
	10	10	19	10	10	32	25
	5	25	25	5	20	27	20
	20	12	24	3	15	40	22

FIG. 14. Analysis of variance of height of each line of regenerated plants.

SOURCE	DF	SS	MS	F
FACTOR	6	5056.4	842.7	26.86
ERROR	98	3075.2	31.4	
TOTAL	104	8131.6		

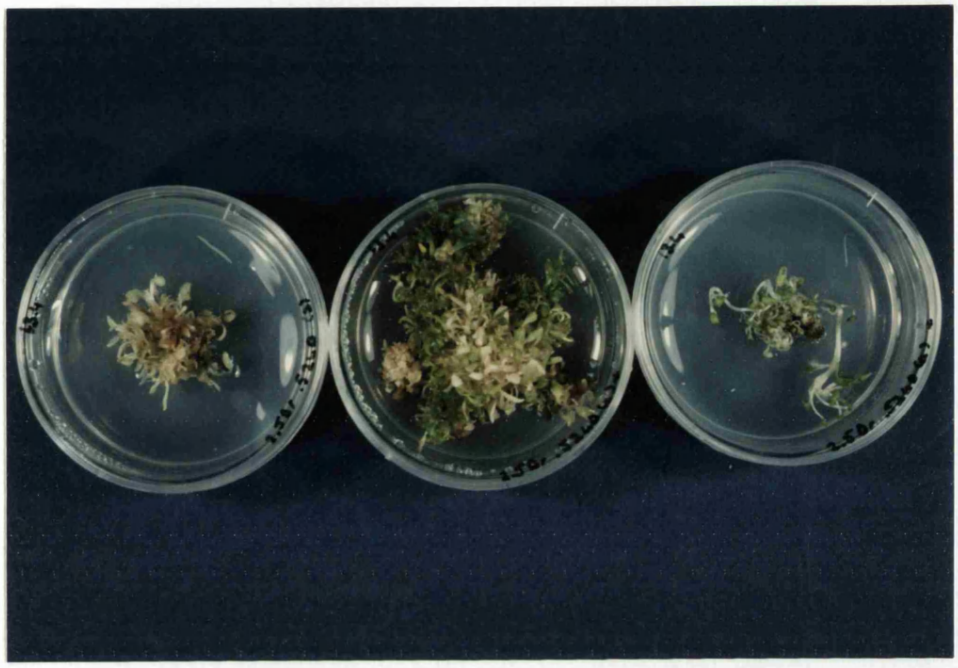
LINE	N	MEAN	STDEV
1	15	12.400	5.792
2	15	14.933	5.298
3	15	24.333	5.447
4	15	7.533	3.681
5	15	20.867	6.278
6	15	29.267	7.015
7	15	21.933	5.120

POOLED STDEV = 5.602



Exp. 20. Introduction of regenerated shoots into the commercial propagation system.

7 variation of regenerated plants in tissue culture

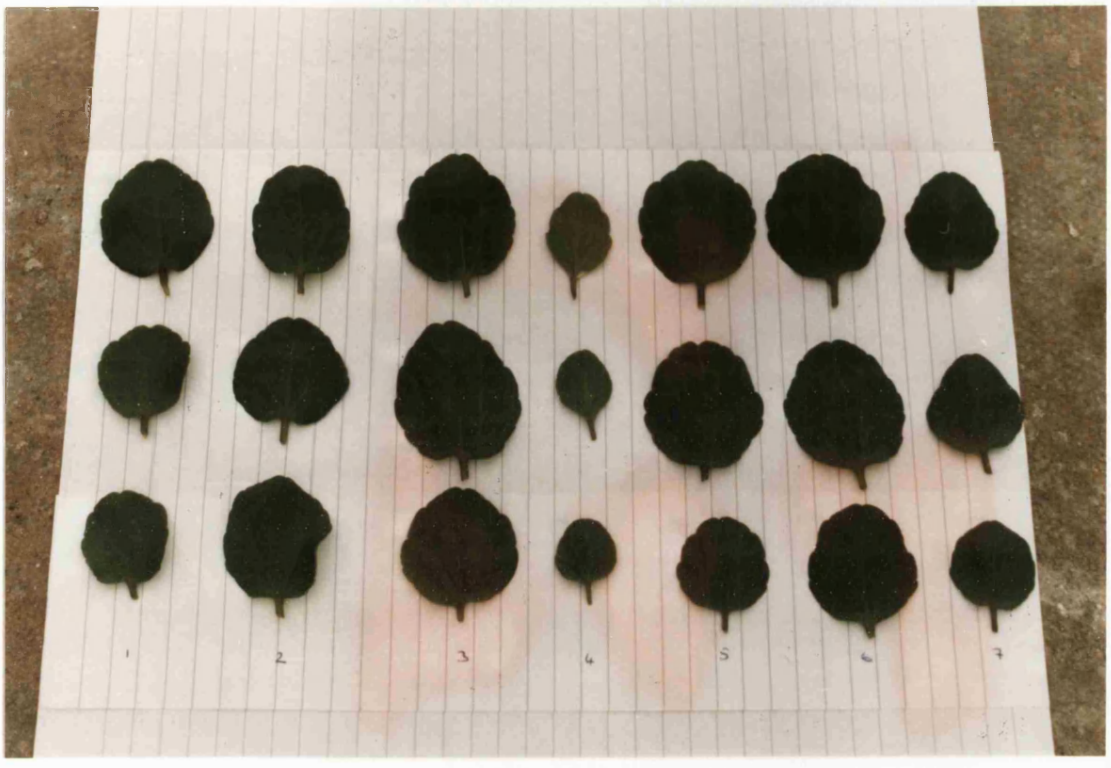


7a variation of development of 3 shoots regenerated from the same callus formed on 2.5µM Dropp and 0.5 µM 2,4D

7b variation of development of 3 shoots regenerated from the same callus formed on 12.5µM Dropp and 0.5µM 2,4D

8 variation of regenerated plants *in vivo*

8a regenerated plants of lines 1 to 7



8b variation in leaf shape of regenerated lines 1 to 7

4. DISCUSSION

The significance of the results obtained in section 3 is discussed.

4.1 REGENERATION FROM CALLUS

Murata & Orton (1987) found that the most important factors for callus initiation were auxin and species. The influence of genotype was also noted by Yeoman & Forche (1980) in 23 oat genotypes where 16 genotypes regenerated plants and 2 genotypes failed to produce callus. Feldmann & Marks (1986) working on *Arabidopsis thaliana* also found differences between genotypes in the percentage of explants that regenerated shoots. In this study there was no noticeable difference between callus formation on the two genotypes tested. However there was a difference between numbers of shoots regenerated on the two genotypes tested in this work which may reflect a genetically controlled difference.

Successful establishment of a callus usually depends on PGRs and nutrients that select for rapid cell division. Murata & Orton (1987) found that 2,4D gave more frequent callus formation compared to NAA in *Brassica alboglabra*. Dietart *et al* (1982) found that for several *Brassica* species 2,4D was necessary for callus formation. However 2,4D is not a universal requirement for callus formation in the *Cruciferae* as Pareek & Chandra (1978) found that 2,4D was not necessary in cauliflower. In this report callus was formed on cytokinin only without the presence of an auxin in the medium. Murata & Orton (1987) found that addition of a cytokinin to an auxin-containing medium, did increase

the frequency of callus formation. Regeneration of shoots has usually been achieved in the *Cruciferae* by transfer of explants to auxin-free medium eg Horak *et al* (1975), Lustinec & Horak (1970). 2,4D may have a residual effect on explants even after it has been removed from the medium. Kale callus initiated on medium containing 2,4D produced fewer shoots on transfer to auxin-free medium than callus initiated on NAA-containing medium (Horak *et al* 1975). The duration of the experiment is not stated but the results for watercress show a similar pattern up to about week 16. In *Brassica alboglabra* (Murata & Orton 1987) either 2,4D or NAA in the regeneration medium inhibited shoot formation. In EXP. 1, to test the effect of PGRs on shoot regeneration, shoots were produced on the initiation medium containing Dropp and NAA. However in the experiment to show the influence of culture variables on shoot formation, continued presence of NAA in the medium was inhibitory to shoot formation. Yeoman & Forche (1980) state that 2,4D tends to suppress morphogenesis and it may be that as it is a long-lived artificial auxin it continues to be present in cultures for a considerable period after its removal from the medium.

Several workers (Fellman *et al* 1987, Mok *et al* 1987) have commented on the potential for use of Dropp in tissue culture systems, as it has a cytokinin activity similar to the most active cytokinins of the adenine

type (Mok *et al* 1982). These results indicate that its use in watercress is promising. In the only previous report of the use of Dropp in the *Cruciferae* the optimum concentration for shoot proliferation in broccoli was 0.3μM compared to 10μM for i^6 Ade. Dropp at 10μM caused abnormal growth and vitrification. There are also few reports on shoot regeneration using Dropp, but in petunia leaf explants shoot bud initiation was profuse at 1μM Dropp while at higher concentrations shoot formation was suppressed and Dropp became toxic (Fellman *et al* 1987). In contrast the optimum concentration of Dropp for watercress was 12.5μM and it may be higher. The mode of action of Dropp is not yet clear, it may act directly at the site of adenine-type cytokinin action (Kurosaki *et al* 1981) or it may influence endogenous cytokinin biosynthesis (Thomas *et al* 1986).

Though the combination of Dropp and 2,4D gave the highest frequency of regenerated shoots, its late peak at 28 weeks must be considered in conjunction with the aim of an experimental system. There is considerable evidence that changes can occur in cultured cells (Griesbach 1987) which have been associated with abnormalities in regenerants e.g. Dunwell (1981) found gross changes in ploidy in shoots regenerated from *Brassica oleracea*. In tobacco the frequency of aneuploid cells increases with age which is associated with loss of morphogenetic potential (Murashige &

Nakano 1967). It is also known that 2,4D causes sister chromatid exchanges (Turkula & Jalal 1985) which if assymmetric, can lead to mutations (Larkin & Scowcroft 1981).

Bayliss (1980) points out that it is difficult to separate the effects of 2,4D in the medium from indirect effects caused by the degree of tissue organization. It has been noted that PGRs can select for cell lines of different ploidy (Shamina 1966). Cells of high ploidy may already exist in the original explant e.g. pea roots Matthysse & Torrey (1967). Culture conditions may select for cells capable of undergoing rapid division and therefore select initially against cells showing metabolic specialisation (Yeoman & Forche 1980). It is known that both NAA and 2,4D can cause spindle failure and mitotic abnormalities at herbicide concentration i.e. greater than 50μg/ml (Bayliss 1980). However at the concentrations normally used in tissue cultures the effect of 2,4D appears to be solely due to the degree of organization. It is suggested that in organized growth *in vivo* or in differentiating cultures the polarized cellular environment ensures the accuracy of mitosis (Bayliss 1980). Larkin & Scowcroft (1981) point out there is probably selection against gross karyotype changes during plant regeneration. There is strong evidence to suggest that chromosomal change occurs as a direct result of growth in culture, but

that the rate of accumulation of variants corresponds with the rate of disorganized growth and cell division, hence the effect of PGR treatments which stimulate disorganized growth (Bayliss 1980). Therefore, for regeneration of particular genotypes, periods of disorganized growth should be limited as much as possible. Thus it would seem that the combination of Dropp and 2,4D would be more suitable for a system for disease resistance selection based on somaclonal variation, whereas, as the combination of Dropp and NAA produces shoots very rapidly, though at a lower frequency, it would be better for the regeneration of plants from microspore-derived callus.

It is clear that attention must also be paid to culture variables other than PGR combination. There was a difference in response between leaf explants and petioles and such differences between different parts of the plant have also been noticed by Johnson & Mitchell (1978). The choice of innoculum is of paramount importance for achieving success in regeneration and aspects to be considered include organ, physiological and ontogenetic age, seasonal variation, size of explant and quality of donor plant (Murashige 1974). Various other workers have reported different responses between different parts of a plant eg Murata & Orton (1987) found differences in shoot formation between cotyledon and hypocotyl-derived explants. Wong & Loh (1987) found different responses

between seedling-derived explants and mature tissues. They also noticed callus formation at the basal end of internodal segments. A similar polarity of callus formation was noticed in petiole explants of watercress.

Culture of explants in the light gave the most notable improvement in shoot regeneration, but other factors also appeared to be advantageous. Gelrite does not contain the impurities known to be present in agar, which are deleterious in some systems (Dunwell 1985b) and its use gave an improvement in callus growth and shoot production. CH is a mixture of compounds and it would be worth attempting to identify which of its components were particularly beneficial. CH has been found to increase the range of auxin and cytokinin concentrations at which shoots could be formed (Thorpe 1980). Consideration should also be given to transfer of calli to PGR-free medium, after a suitable period of initiation, to remove any toxic, mutagenic or selective effects of the PGRS. The lack of significant difference between levels of Dropp in EXP. 2, to investigate the effect of various culture variables was probably because its effect was swamped by the effects of the culture variables.

Most of the papers dealing with organogenesis fall into the manipulation category. The understanding of the mechanism of organogenesis is limited because of the

lack of biochemical or physiological studies. One major drawback is the lack of a suitable experimental system as only a few cells in an explant are directly involved in organ initiation (Thorpe 1980). It would also be valuable to understand mechanisms for somaclonal variation, because if it could be controlled, it could be enhanced when required or suppressed if propogation of useful genotypes is intended (Larkin & Scowcroft 1981).

Regeneration of plants via callus may have commercial applications in several systems. Wong & Loh (1987) are investigating selection for disease resistant variants of *Brassica alboglabra* and also transformation with *Agrobacterium tumefaciens* and *A.rhizogenes*. Feldmann & Marks (1986) are also working on development of a transformation scheme with *Agrobacterium* for *Arabidopsis thaliana*. Sacristan (1982) has produced plants resistant to the pathogen *Phoma lingam*, by regeneration from callus and embryogenic cultures of haploid *Brassica napus* previously treated with mutagens. Two selection techniques were used, one where cultures were treated with spores and one where selection was on a medium containing toxic filtrate of the fungus. It is hoped that similar techniques will be used for selection of lines of watercress resistant to crook root disease, once regeneration rates have been raised to a suitable level by optimising all culture variables.

4.2 REGENERATION FROM ANTHERS

4.2.1 Testing of procedure using oilseed rape

Dunwell & Cornish (1985) using the 2,4D medium found an average of 26.9% induced buds for 'Duplo' with a maximum of 43.4% induced buds for 2mm long sequentially harvested buds. Clearly in the experiment to test anther culture of the model plant the percentage of induced buds is much lower, except for buds cultured on the 2,4D and Gelrite medium. This treatment however was not carried out on a statistically significant sample size. These workers also found an average of 2.02 induced anthers per induced bud which is lower than the average value of 2.63 induced anthers per induced bud found in this experiment, but Dunwell & Thurling (1985) found 3.17 induced anthers per induced bud using the 2,4D medium and 8% sucrose. Dunwell et al (1983) using the BA medium found 11.4 embryos per induced bud for Duplo which is slightly higher, but in the same range as the 10.3 embryos per induced bud on BA in this experiment. The overall average for embryos per induced bud obtained in this experiment was 11.6. Dunwell et al (1983) also found 2.55 embryos per cultured bud for Duplo on BA medium which is considerably better than the 0.89 embryos per cultured bud found in this experiment, and 28.6% induced anthers compared to 4.39% induced anthers in this experiment.

It should be noted that experiments in all the reports mentioned above were carried out on liquid medium and that no embryos were produced on liquid medium in this experiment. This may have been because the anthers sank and became anaerobic in this experiment. Anthers float on liquid medium due to the combination of density and surface tension effects. If the anthers were more dense than the medium they could still be made to float by surface tension forces. These forces can be broken by turbulence such as that caused when moving plates to an incubator at a lower temperature, in which case the anthers would sink. This problem could be overcome by culture of anthers in one incubator or by use of filter paper bridges; very thin layers of medium or increasing the medium density. Prakash & Giles (1987) also found solid medium to give better results than liquid medium for *B. napus*. Agar is known to contain impurities which may be deleterious to anther culture (Dunwell 1985b) and this may explain the low percentage induced anthers and induced buds found in this experiment, and the differences in numbers of embryos per cultured bud. Numbers of embryos per induced bud and numbers of induced anthers per induced bud were in the same range in this experiment and the reported experiments, so this aspect of yield of anther culture, may not be affected by the deleterious effects of the agar. Dunwell (1985a) suggests that the two aspects of yield i.e. percentage

of anthers producing embryos and the number of embryos produced per anther are determined independently.

Dunwell & Cornish (1985) found that 2mm buds gave the highest number of induced buds and this corresponds with the finding in this experiment that most of the embryogenic buds were 2mm in length.

No cytological examination of the regenerants was carried out in this work but those plants which had small sterile flowers and did not set seed were probably haploid, The remaining plants were probably diploid though ploidies of up to hexaploid have been reported (Sunderland & Dunwell 1977). The majority of these non-haploids are homozygous and have arisen as a result of chromosome doubling during culture (Dunwell 1985b). In some species non-haploid pollen does occur at a low frequency (Dunwell 1985a) and this would give rise to heterozygous regenerants. However this has not been reported in the *Cruciferae*.

4.2.2 Effect of gelling agent on anther culture of oilseed rape

The experiment to investigate the effect of gelling agent on anther culture of oilseed rape var Duplo was carried out to investigate more fully the result obtained in the earlier experiment (EXP. 5) in which the highest frequency of embryo production was on a

medium solidified with Gelrite. The recommended level of Gelrite in plant tissue culture media is 2gdm^{-3} (Kelco 1986a) so the range chosen for test included two levels below this and one higher. For agar the usual concentration used in plant tissue culture media is 7gdm^{-3} so again the range tested included two levels below this value and one above. Past experience indicated that levels of gelling agent below the minima for this experiment would not produce a satisfactory set of the media.

These results indicate that for this cultivar of oilseed rape, the gelling agent is significant and that Gelrite, particularly at the lowest level tested is a notable improvement over agar. Since Gelrite is the product of a tightly controlled fermentation process (Kelco 1986a,b) it is likely to be consistent in quality and have a high level of purity. Agar is a natural product which may contain impurities. Debergh (1983) found that aqueous extracts of homogenized agar gels contained more than 100% of the macro elements introduced in the basal medium, and that considerable amounts of Cu^{2+} were present as an impurity in agar gels. The impurities present in agar are known to be inhibiting for embryo formation from anthers (Dunwell 1985b) so this may explain why Gelrite gave better results for embryo formation in this experiment.

Tests on different concentrations of agar showed that agar concentration had a significant effect on the concentration of Ca^{2+}, K^{+}, Na^{+}, Mg^{2+} + Mn^{2+} in the medium (Debergh 1983). There was also a gradient of these ions within the gel which varied with the agar concentration. This may have been brought about by evaporation of water from the agar surface, the rate of which varies with concentration. Water stress affects growth and development of plants in tissue culture. There is preliminary evidence for globe artichoke (Bouniols 1974) that there is a 20% increase in proline content of cultures when the concentration is raised. Proline content is often used as an indicator of stress. The availability of water is influenced by the solidity of the gel and the type of gelling agent (Debergh 1983). It would seem reasonable therefore that differences in nutrient availability and reduced availability of water could explain the low rate of embryo production on the higher concentrations of both agar and Gelrite.

It should also be considered that use of Gelrite had a beneficial effect on anther culture as it is used at a much lower concentration than agar i.e. 0.2% compared to 0.7% for agar.

It may be that the usual recommended levels of these gelling agents are not optimal for all types of tissue culture system, and consideration should be given to

this easily controlled medium component when commencing work on any tissue culture system.

4.2.3 Regeneration from watercress anthers

The results of pollen staging proved that some guidance to the stage of pollen development can be obtained from morphological characters such as bud length or petal length:anther lengthratio, and since bud length is easier to measure this character was chosen for use before anther culture. Other workers have tried to stage pollen further by removal of the exine, but there are no enzymatic methods of removing sporopollenin and because it is so chemically inert, chemicals methods to remove the exine are very destructive. Dunwell *et al* (1983) found staging after exine formation to be impossible. It must be noted that there may be differences between clones in the exact stage of pollen development for a given bud length. The relationship may also vary with the age of the donor plant. However Thurling and Chay (1984) also concluded that bud length is a good indicator of distribution of pollen stages.

The results of regeneration from anthers of watercress indicate that the genotypes of watercress that were tested do not undergo direct embryogenesis in anther culture. The anther-derived callus and two small globular structures on anthers of J1 and J7 may have been of microspore origin but this was impossible to

determine. It must be realised that even if these were of microspore origin such infrequent events are of no value in a crop improvement programme. Production of microspore callus was also limited in its applications by lack of an efficient plant regeneration protocol at the time these experiments were carried out and this also proved to be the limiting step in anther culture of *Iberis amara* (Babbar *et al* 1980).

There are many factors which influence whether a plant will respond positively to anther culture. The most important of these is genotype and Dunwell (1985c) points out that the constraints imposed by choice of genotype may severely limit the applications of anther culture. Even in responsive species large numbers of anthers may have to be cultured to detect a small response. Wenzel *et al* (1977) working with six cultivars of *Brassica napus* found a maximum response of 1.8% which corresponded to 34 androgenic anthers out of 19,422 cultured, and with some media they cultured 8,892 anthers to find no response. In maize, where Bretell *et al* (1981) found 22 responsive anthers out of 21,638 cultured, it is pointed out that the large input of manipulative labour required is a severe limitation for application of anther culture techniques. Another drawback is that plants which should be expected to give a good response often give a poor or zero response (Ockendon 1985). However culturing large numbers of anthers on an occasion may not necessarily improve the

consistency of the results as the more anthers cultured, the greater the risk of damage during excision which would give lower embryo yields (Ockendon 1985). Thurling & Chay (1984) found that both donor plant genotype and its growth environment affected the production of multicellular microspores in *Brassica napus* ssp *oleifera*. A similar finding has been made in wheat where the optimum temperature for culture of anthers from field grown material is about 2°C higher than for greenhouse grown material independent of plant genotype (Ouyang *et al* 1987). Thurling and Chay (1984) emphasize the importance of modifying the growth environment of donor plants, but point out that this may not be universally successful in promoting pollen embryogenesis. Evidence for the genetic control of response to anther culture has been found in a study of eight inbred lines of *Brassica oleracea* var. *gemmifera* and ten F_1 hybrids derived from them (Ockendon & Sutherland 1987). Half the total variation in embryo yield was calculated to be genetic and there was partial dominance for this character. The relatively high heritability of responsiveness means that it may be possible to introduce this character into non-responsive lines.

Numerous attempts have been made to extend the technique of androgenesis to the rest of the angiosperms and gymnosperms but without much success. In most responsive species judicious adjustment of the

pollen stage, medium and PGRs have resulted in a reasonable level of response. A number of factors have been identified which are thought to interrupt normal pollen development allowing the microspores to follow a sporophytic developmental pathway. These factors include thermal shock (Keller *et al* 1975), cold pretreatment (Lichter 1982), ABA and mannitol (Imamura & Harada 1980), reduced atmospheric pressure and anaerobic environment (Harada & Imamura 1983). However none of these treatments have been effective in inducing androgenesis in various recalcitrant species. Sangwan & Sangwan-Norreel (1987) believe that the pollen of some species is genetically predisposed to follow an androgenic pathway while pollen of recalcitrant species is not. Most reports of techniques for anther culture are empirical and are not based on any detailed comprehension of pollen physiology or biochemistry (Dunwell 1985b). This is mainly due to the difficulties in identifying the embryogenic pollen as only a small percentage of the pollen (up to 8% in *Datura*) usually undergoes embryogenesis, though in some species nearly all the pollen can be induced to undergo one or a few divisions. However a specific cytological marker has been found (Sangwan & Sangwan-Norreel 1987) allowing identification of embryogenic pollen after only 12 hours. Embryogenic pollen develops a specific thin-layered tannin coating on the tonoplast. It is important to determine whether it is normal pollen or

abnormal pollen which undergoes embryogenesis. It has been suggested that it is the latter for several reasons:

1. If embryogenic pollen grains were normal then improved media and culture conditions should have resulted in considerably increased percentages of responsive pollen grains.

2. The existence of pollen dimorphism e.g. in *Nicotania tabacum* two distinct staining classes of pollen in acetocarmine can be recognised (Sunderland & Dunwell 1977). The fact that highly embryogenic pollen fractions can be separated by centrifugation (Maheshwari *et al* 1982) may also show dimorphism. However it has also been suggested that centrifugation may be acting to disrupt the microtubule system (Hu & Huang 1987) but Vasil (1980) states that centrifugation does not give an increase in the number of embryogenic microspores. It has been pointed out however that the number of embryogenic pollen grains can exceed the number of abnormal pollen grains (Vasil 1980) which must mean that normal grains can undergo embryogenesis.

3. Culture media and conditions do not affect the early embryogenic pathway. In *Nicotiana* and *Datura* a switch to embryogenic development has been

achieved in distilled water and 2% sucrose (Vasil 1980). This would support the hypothesis that some pollen is predetermined to be embryogenetic.

4. The observation of abnormal cleaving of PMCs into microspores in which cytoplasmic abnormalities could be seen and the hypothesis that these still contain sporophytic long-lived mRNA which may interfere with gametophytic development of the pollen (Sangwan & Sangwan-Norreel 1987). It has been reported that ABA can inhibit RNA synthesis and may block synthesis of mRNAs necessary for gametophytic development so triggering a switch to sporophytic development (Villiers 1968). This is also supported by the observation that treatment of anthers of *N. tabacum* with ABA for 3 days increases embryogenic responses.

Assuming that the callus produced in these experiments is of microspore origin&thus means a responsive genotype has been found a number of factors have been found to influence the frequency of response. The orientation of the anther has been found to be important in barley (Shannon *et al* 1985), maize (Tsay *et al* 1986), rice (Mercy & Zapata 1987) and in *Brassica* (Dunwell 1985b) where it is recommended that the outer flat side of the anther is placed in contact with the medium. It was noted in the results that the response of anthers from the same bud could be markedly

different and it is proposed that this could be due to differences in anther orientation. The effect of orientation may be of particular importance in small anthers because the surface tension-induced film of water around the anther is large in relation to its size (Shannon *et al* 1985). Another possible explanation of the difference in response between anthers is that the excision process is slow and the sixth anther may therefore be more dehydrated or otherwise damaged than the first. There is some evidence that ethene (ethylene) influences the response of anthers. Dunwell (1979) found that for anthers containing pollen at a stage after the first pollen grain mitosis, in *Nicotiana tabacum*, removal of ethene increased the survival of embryos, and there was also an effect of volume of culture vessel atmosphere on embryo production. There was a marked difference in response to ethene removal with increasing anther age and it was concluded that ethene is implicated in embryogenesis. In contrast, Reynolds (1987) found in bicellular pollen grains *Solanum carolinense* L. that IAA induced pollen embryogenesis and it is suggested that it acts through auxin-mediated ethene production. Embryogenesis was also increased by Ethrel and the ethene precursor aminocyclopropane carboxylic acid, though to a lesser extent, so it appears that the auxin also has a direct effect on embryogenesis. High levels of Ethrel (0.01%) or Ethrel plus IAA were found to be inhibitory to anther culture. Ethene production is

associated with wounding of plant tissues, so varying degrees of damage and hence ethene evolution may also explain differences in response between anthers of the same bud..

In *Nicotiana tabacum* the known ethene antagonist silver nitrate ($AgNO_3$) (Beyer 1976) gave a marginal increase in yield of pollen plantlets but this was not regarded as an efficient means of increasing productivity (Dunwell 1979). The role of ethene in anther culture of *Brassica oleracea* var. *gemmifera* (Brussels sprout) was investigated by testing various levels of $AgNO_3$ (Biddington *et al* in press). The results showed that $AgNO_3$ acted as a promoter of embryogenesis in cultivars that are normally poorly responsive to anther culture. It acted over a wide range of concentrations (0.1 - 10$mgdm^{-3}$) and was only inhibitory at one concentration for one cultivar in one of sixteen experiments. It is suggested that in Brussels sprouts, $AgNO_3$ promotes embryogenesis by blocking the inhibitory effect of endogenous ethene on embryo production and that genotypes which are normally highly responsive either produce less endogenous ethene or are less sensitive to its effects. These reports indicate that ethene is implicated in the switch to sporophytic development and there may be an optimum level of ethene above which its effect on embryo production is inhibitory. It has been noted that rates of respiration and of ethene evolution of bicellular and tricellular pollens differ

considerably so different types of atmosphere may be necessary to trigger sporophytic development (Sunderland & Dunwell 1977). This may explain the interspecific differences in response to ethene or its removal, reported above.

Presumably other auxins also act to stimulate endogenous ethene production which may explain why the tricellular pollen families such as *Cruciferae* and *Graminae* require an auxin in most cases for successful anther culture (Dunwell 1985a), though there is a report of successful production of microspore-derived embryos, from isolated pollen in the *Cruciferae* without use of PGRs (Swanson *et al* 1987). It may be that isolated pollen has different PGR requirements to cultured anthers. There may also be differences in PGR requirements between the bicellular pollen families e.g. *Solanaceae* and the tricellular families, as several solanaceous species can be induced to undergo embryogenesis without PGRs (eg Nitsch 1981).

Sucrose has been used in most studies as a carbon source for anther culture and research has been carried out into its optimum concentration. Dunwell & Thurling (1985) found that high sucrose concentrations (16% and above) reduce variation in response between cultivars of oilseed rape. It is suggested that this concentration is closer to the osmotic pressure within the anther and that lower sucrose concentrations select

against those cultivars with anthers less able to withstand osmotic stress. It may also be that high temperature treatments act by increasing evaporation of water from the medium and therefore increasing osmotic pressure. Imamura & Harada (1980) found that water stress on anthers of *Nicotiana tabacum* caused by addition of 0.5M mannitol to the medium had a stimulatory effect on plantlet formation, where the control response was low. ABA at 10^{-5}M also had a stimulatory effect and water stress was also found to increase the level of endogenous ABA. This finding may be linked to the action of high sucrose levels. However there is some evidence that lower concentrations of 8-10% sucrose may be better for continued embryo survival (Dunwell 1985). Another aspect of medium composition which has rarely been considered in conjunction with anther culture is the hydrolysis of sucrose during autoclaving which leads to an increase in osmotic pressure (Dunwell & Thurling 1985). Autoclaved sugars may stimulate growth in tissue cultures more than filter sterilized sugars (Romberger & Tabor 1971). The nature of the sugar may also have an effect on the response to anther culture. A recent study in barley (Hunter 1987) has shown that there is still scope for manipulation of the sugar component in the medium and oligosaccharides and polysaccharides containing at least 2 glucose residues are particularly effective in improving yield. However in *Brassica campestris* (Keller *et al* 1975) 10% sucrose

was found to be optimum, 15% sucrose gave a lower response and glucose, maltose and raffinose gave no response. Lichter (1981) found that 7% sucrose + 1% glucose gave a lower response that 8% sucrose in liquid culture of *Brassica napus* anthers. Therefore the limited available evidence suggests that sucrose is the best carbon source in the *Brassica* species, but the optimum concentration should be established for a new species. In this study a range of sucrose concentrations were tested ie 2, 5, 10 and 15% w/v but as there was very little response the optimum concentration could not be determined.

In conclusion it seems that there are three possible explanations for the failure to produce embryos or callus of microspore origin.

1. The appropriate medium, thermal shock and PGR combination have not been found. In reported successes of anther culture, some response has usually been found on suboptimal media. If the calli produced on J1 and J7 do represent a low frequency response then further modification of the medium should increase this response.

2. A genotype with the appropriate genes for response to anther culture has not yet been found, but may be located by further study.

3. The gene combination for androgenesis is not present in watercress. It is notable that out of 28 genera in the *Cruciferae* that are economically important as crops (Macleod 1976) successful anther culture has only been reported in 7 genera consisting of various *Brassica* species. It has also been reported in *Arabidopsis thaliana* and haploid callus formation in *Iberis amara* (an ornamental) has been reported. Either anther culture has not been attempted in the other species or has been attempted but failed and therefore has not been reported.

It also seems that such a project as the anther culture of watercress requires a larger input of labour and time than is available within the restrictions of this type of research project, especially considering the amount of relevant background research which had to be carried out to make anther culture possible.

The lack of understanding of the biochemical and physiological mechanisms underlying embryo formation from microspores and their control by PGRs is a severe handicap in a study of this kind as experiments can only be empirical. Perhaps when the mechanisms and controls have been elucidated in very responsive species such as *Nicotiana tabacum* this information could be used to design experiments on a more systematic basis.

4.3 REGENERATION FROM POLLEN

Swanson *et al* (1987) reported a high yield of 700-1000 embryos per bud (120-170 per anther) for isolated pollen culture of *Brassica napus* L. var. Topas. Yields this high have not been reported for any other species or cultivars. Lichter (1982) reported an average yield of 1.6 embryoids per anther for *Brassica napus* var. Tower though the maximum yield was 3.4 embryoids per anther at the optimum sucrose concentration of 3.5M. Chuong & Beversdorf (1985) reported improved yields of between 7.0 and 9.6 embryos per anther for six cultivars of *Brassica napus* and 54.1 embryos per anther for one cultivar of *Brassica carinata*. These reports all indicate the potential for production of microspore-derived plants by isolated pollen culture in the *Cruciferae* but no clear guidelines for various aspects of culture have yet emerged.

Chuong & Beversdorf (1985) found that some cultivars have very precise temperature requirements e.g. Isuzu produced 9.6 embryos per anther at 30°C but only 1.6 embryos per anther at 32°C. Most of the cultivars gave a maximum embryo yield at 30°C but CR-1 gave a maximum yield of 9.4 embryos per anther at 32°C. In contrast Lichter (1982) reported a yield enhancemement by pretreatment of the flower buds at 4°C. The culture

temperature seems also to have an effect on the embryo development. In *Brassica carinata* at 25°C, 70% of embryos appeared normal compared to 19% at 32°C. A split temperature regime of 32°C then 25°C was suggested to compensate for low yields at 25°C (Chuong & Beversdorf 1985). Swanson *et al* used 30°C initially and then 25°C after 12-14 days.

The earlier papers (Lichter 1982, Chuong & Beversdorf 1985) include use of PGRs, BA and NAA in the medium and Chuong & Beversdorf ascribe their increased yield and dispensation with the need for a low temperature pretreatment to increased levels of NAA. They used $1mgdm^{-3}$ compared to Lichter's $0.5mgdm^{-3}$ (1982). However Swanson *et al* (1987) did not include any PGRs and achieved a very high yield, which may indicate that their effect is inhibitory. In both *Datura innoxia* and *Nicotiana tabacum* PGRs are unnecessary for successful pollen culture (Sunderland & Dunwell 1977). Kameya & Hinata (1970) using 7 species of *Brassica* succeeded in obtaining pollen derived calli from two species when culturing isolated pollen in a medium containing coconut milk, but could not raise shoots from these calli.

In spite of the limited information available isolated pollen culture has a number of advantages over anther culture, which makes the technique attractive.

The efficiency of isolation of pollen is independent of bud size and this is a considerable advantage over anther culture, particularly for a small budded species like watercress. Anther removal is time consuming and laborious in watercress. The small flower buds are also vulnerable to damage during dissection and this may lead to considerable variation in the viability of pollen within the anther. In isolated pollen culture the extraction is standardized for all buds. Washing procedures are carried out immediately to reduce any deleterious effects of proteases or other inhibitors released from the damaged somatic tissue (Swanson *et al* 1987).

The technique is also useful in watercress as it would be possible to carry out a wide screening programme of genotypes in a relatively short time and to test a range of bud sizes in adequate numbers. One problem with this research into anther culture of watercress has been the impossibility of culturing sufficiently large numbers of anthers of any line, in the time available, to detect any small response.

Hu & Huang (1987) suggest that albinism is more of a problem in pollen culture than in anther culture, but that early excision of the pollen gives more green plants. Though albinism has not been reported in pollen culture in the *Brassica* species, use of pollen

is free from limitations of the bud size that can be handled, so earlier excision should be possible.

There is some evidence to suggest that haploids are more prevalent in the regenerants from pollen culture compared to anther culture eg in *Datura innoxia* 99% of embryos derived from pollen culture were haploid compared to 41% of embryos from anther culture (Sunderland & Dunwell 1977). This may be considered an advantage, as the probability that haploids are of pollen origin is very high but this would depend on the aim of the experimental system.

A particularly important aspect of the development of an isolated pollen culture system is its potential use in mutation and *in vitro* selection studies. A recent paper (Swanson *et al* 1988) reports the first use of mutagen-treated isolated microspores for selection of increased tolerance to a herbicide Chlorosulfuran (CS). Resistance was selected for by treating microspores with one or two mutagens and then culturing in a medium containing 3 ppb CS. Two plants were regenerated which showed increased tolerance to CS.

Such a system has several advantages in that it is based on a relatively synchronized population of haploid single cells. Deleterious mutations are rapidly selected against without a series of back

crosses and selected haploid mutants can readily be incorporated into a breeding system as homozygous lines.

So far isolated pollen culture has only been successful in a few species and the technique is often considered to be very difficult (Sangwan & Sangwan-Norreel 1987). However the advantages mentioned above and the possibility of using the technique for a better understanding of the biochemical control of embryo development from microspores make its use attractive for further study.

4.4 REGENERATION FROM OVULES

Haploid plants have now been produced by ovary and ovule culture in a number of species eg rice (Zhou & Yang 1981), barley (San Noeum 1976), wheat, tobacco and *Gerbera* (Yang & Zhou 1982). Data are limited perhaps because of the relative ease with which haploids have been produced from anthers, so that studies on the megaspore have seemed unattractive. However in at least one species *Beta vulgaris* L. ovule culture is considerably more efficient for production of haploids than other methods of haploid production (Bossoutrot & Hosemanns 1985). There are various problems associated with ovary or ovule culture such as assessing the developmental stage of the embryo sac and this has usually been assessed indirectly by staging of pollen e.g. San Noeum (1976). However unlike pollen culture a wide range of developmental stages seem to be able to respond to ovule culture including nearly mature stages. Keller *et al* (1987) point out that a major disadvantage of ovule culture is that even if all ovules could be induced the embryo yield would be much lower than for anther culture, as the number of ovules is always small compared to pollen.

Media recommended are similar to those used for anther culture (Dunwell 1985c) and most successful experiments have used solid media. There are no reports of the use

of ovary or ovule culture in the *Cruciferae*. No general guidelines for culture conditions can yet be obtained from the literature, but culture of the megaspore does seem to have some advantages over anther culture in that albinism occurs less frequently and the regenerants may be more genetically stable than anther-derived regenerants (San Noeum & Ahmadi 1982).

Development has been reported via callus or direct embryogenesis. In this experiment two calli were produced from ovules, but as in anther culture, there can be a problem in distinguishing the origin of the callus as somatic cells may also proliferate. A further problem is that production of callus means that regeneration of shoots has to be carried out and this was the limiting step in this experiment. The fact that two calli were produced from 48 buds tested and one turned green is initially more promising than the results obtained for anther culture in watercress, but further improvements in efficiency of the plant regeneration protocol from callus for watercress are vital before further effort is directed at ovule culture.

4.5 LONG TERM STORAGE

Survival in culture varies considerably between species. El-Gizawy & Ford-Lloyd (1987) found no survival at 4 months in garlic while in potato (Westcott 1981a) up to 14% of cultures survived for 12 months and Kartha *et al* (1981) found that *Coffea arabica* L. in shoot tip culture could be stored for up to 2 years without special treatments. In comparison 19% of Clone A control shoots and 29% of J1 control shoots survived after 12 months storage.

There is clearly a difference in response between the two clones and while 1% mannitol appears to be a useful treatment in both clones, the use of 5mgdm^{-3} ABA would only be advantageous in clone A. It would seem therefore that it would be necessary to carry out tests to establish the optimum treatment for any line that was to be stored in tissue culture.

Slowing of the metabolic rate by general mechanisms such as the osmotic stress caused by increased mannitol levels, or low temperatures should increase longevity by increasing the period before medium components become exhausted and therefore growth-limiting. It may be that exhaustion of the carbon source is the cause of death in cultures over a period, so that increasing amount of carbon source such as sucrose in the medium

might increase the longevity of the culture. However it would appear, in watercress, that this permits more rapid and larger growth in the first couple of months of culture, which probably exhausts other medium components more rapidly than normal, so death occurs relatively early. Growth retardants such as ABA and paclobutrazol are presumed to act by reducing internode length so that growth is limited and the time before the medium becomes exhausted is increased. However this effect may be outweighed by production of larger leaves, as appeared to be the case for the higher ABA concentrations and for paclobutrazol at 1 and 5 ppm. The highest level of paclobutrazol was probably almost completely inhibitory to gibberellin synthesis, a condition which the shoot could not survive.

Of the treatments tested in these experiments for long term storage of shoot tip cultures, only storage at 10°C showed a sufficiently marked and consistent improvement for both clones to be considered suitable for use in a commercial storage system. It is possible that even lower temperatures may be more effective for storage. Marino *et al* (1985) found that *Prunus* rootstocks could be stored *in vitro* at 4°C and -3°C for 200 days. Use of below freezing temperatures would depend on the frost tolerance of the plant. Watercress does suffer from freezing damage (Rothwell 1983) and is protected in the field by use of borehole water which is at a constant 10°C all year round. Its tolerance of

temperatures between 0 and 10°C would have to be investigated. Another factor that can be important in successful storage is the light regime. In *Prunus* (Marino *et al* 1985) 16 hour photoperiods are beneficial for storage at 4°C but complete darkness is best at -3°C. This may be because at low temperatures light causes damage, such as photolysis, more rapidly than can be made good by the plant's repair mechanisms.

The application of low temperatures to storage commercially may be limited by practical considerations such as the expense of refrigeration equipment, hence the attractiveness of the additions to the medium which were tested here, as they have permitted minimal growth storage of other species in culture without use of expensive equipment. Unfortunately such treatments have not proved to be effective in watercress.

It must be noted that while shoot tip cultures are considered to be relatively genetically stable compared to callus, somaclonal variation may be introduced to the system by adventitious shoot formation (El-Gizawy & Ford-Lloyd 1987). A number of adventitious shoots were formed in nearly all the watercress cultures in this experiment so that the genetic stability of stored cultures could not be guaranteed. In contrast, Henshaw (1982) regards multiple shoot formation during storage to be beneficial, probably because this has the effect of replenishing cultures as they are being stored. In

these experiments the growth of weaned plants was very uniform and no variation was observed which could be attributed to somaclonal variation (though somoclonal variation may still be present).

Survival of the weaning process is important in cases where it is necessary to grow a plant on to flowering, for example to provide material for anther culture, but this aspect of the effect of long term storage in tissue culture seems to have been largely ignored in the literature except that Westcott et al (1977) point out that potato cultures are transferred to soil more successfully if establishment of an effective root system is achieved before transfer. Watercress is very easily transferred to soil, as it develops both basal and adventitious root systems in culture. Both root systems can be used by the plant to take up moisture and nutrients immediately on transfer to soil. A 95% success rate of weaning has been achieved for transfer of 4-6 week old tissue cultured shoots to soil (section 2). Only 63% of shoot tips of clone A surviving for 6 months in culture were successfully weaned, though the reason for this is not clear. However 88% of surviving shoot tips of clone A on 5 $mgdm^{-3}$ ABA survived weaning, so this may be an important aspect for consideration in choosing a long term culture treatment.

4.6 ASPECTS OF THE BIOLOGY OF WATERCRESS *IN VIVO* WORK

The results of the investigation into self-compatibility in watercress indicate that there is no significant reduction in seed set as a result of self-pollination in Clone A, or as a result of the different growth conditions of the two sets of plants. One mechanism by which cross-pollination could be favoured in the field would be by poorer seed set from self pollen. It is possible that Clone A is a particularly vigorous seed setter and its seed production has been reduced from normal, so if facilities had permitted this would have been tested with other self-pollinated lines. The results show that self-pollination can readily occur and that there are no physical or temporal barriers to self-pollination. Crawley (1986) states that one mechanism by which cross-pollinated seeds would be favoured in a population is by having a higher germination rate than self-pollinated seed, but this does not seem to be the case for watercress. It is also an indication of whether watercress is affected by inbreeding depression, which can affect the success of anther culture e.g. if lethal recessives are present.

It is clear from the results in EXP. 19 that clonal seed parents can have a marked effect on the F_1 generation as a crop. In particular plants from y

showed an improvement in some important commercial characteristics. The slight improvement in number of marketable stems of y, though not significant, may still be important in such a high value crop as watercress, if it is consistent. The improvement in plant height was clearly linked with longer internodes. This should mean that plants will reach cropping height more rapidly and hence crop turn around times would be shortened. This is clearly of commercial value as more crops can be grown in a year.

It was noticed that there was a positional effect on plant height with the plants in section 1 at the top of the bed being shorter. Plants tend to develop more sturdily and shorter when disturbed and the water flow is turbulent when it enters the bed at the top, which probably causes sufficient disturbance to produce shorter plants. There is also likely to be a gradient of nutrients down the bed as they are taken up by plants. Though sufficient nutrients are injected into the water supply for the level to be adequate for plants at the bottom of the bed, plants higher up may still benefit from the greater nutrient availability, hence the taller plants in section 2.

Leaf shape is another important commercial aspect of watercress as customers find round leaves appealing. The ratio of length to width can only give an approximate guide to the shape of the leaf i.e. it

cannot distinguish more pointed triangular shaped leaves from round ones, but a difference in leaf shape from the control was found for both x and y. Both x and y were originally chosen because they had visually distinct leaf shapes and these results tend to support the suggestion that the leaf shape has been inherited through a seed generation. Unfortunately no analysis had been carried out on the parent plants when they had been bulked up by tissue culture. If this had been carried out it should have been possible to determine the narrow scale heritability of the leaf shape and thus determine whether the parent plants were self-pollinating, and already highly homozygous. If the plants were heterozygous then segregation would have led to considerable variation in characters such as leaf shape in the F_1 generation. There was no restriction of pollination of the clonal parent plants i.e. they were not grown in isolation.

The results tend to support the theory that watercress is predominantly self-pollinating and would therefore already consist of a number of homozygous lines, though this cannot be proved without a considerable amount of further work. Self-pollination is known to be typical of plants that colonize highly disturbed niches (Lloyd 1980) and running water must be a good example of a disturbed niche. It must be considered, though, that in a watercress bed which consists mainly of homozygous lines, the closeness of neighbours means that cross-

pollination must occur fairly frequently by chance, allowing heterozygosity to remain in the crop.

The results given in EXP. 20 show that it was possible to introduce 7 out of 10 lines of regenerated shoots into the commercial propagation system. There were differences between the lines in the number of shoots that were produced during the *in vitro* "bulking up" period with line 10 failing at this point. Lines 8 and 9 produced noticeably fewer shoots that 1 to 7. The failure of lines 8 and 9 to establish *in vivo* and the significant variation in establishment in lines 1 to 7 indicate that there are differences between the regenerated plants. There were also visible differences in aspects of watercress morphology and a highly significant difference in plant height between the regenerated lines. As all the plants were regenerated from a single plant i.e. all explants were initially the same genotype, the differences between the regenerated plants is probably due to somaclonal variation which occurred during culture, as culture conditions and growth conditions were identical for all plants. Differences in ability to establish *in vivo* may be due to different photosynthetic abilities, changes in surface wax and hence ability to resist desiccation or changes in ability to form adventitious roots.

Watercress is usually very easily weaned for two main reasons:

1. It readily produces both basal roots and adventitious roots at the internodes in tissue culture. Both root systems can act to take up nutrients and the adventitious roots rapidly root into the compost.

2. Watercress roots are adapted to growing in water, due to an aerenchyma system, so that waterlogging is not a problem when keeping humidity levels high.

Rooting and establishment of tissue cultured plantlets is the final stage in the process of micropropagation or regeneration and is of vital importance for production of healthy, high quality material and also so *in vivo* assessments can be carried out. Rooting can be labour intensive and in some species it is difficult to produce a root system with complete vascular connections (Langford 1987). Debergh & Maene (1981) suggest that *in vivo* rooting is preferable as *in vitro* roots may not develop root hairs and there is a cessation of growth on transfer to soil while normal roots develop. However they also point out that plants which develop aerial roots in culture can be rooted directly into compost without a growth stop, as was the case for watercress. Tissue culture-derived cuttings are easier to handle and therefore cheaper than rooted

shoots from tissue culture. The latter may be prone to root damage on transplanting which may allow entry of phytopathological organisms (Maene & Debergh 1983).

Nothing is known about the photosynthetic ability of watercress *in vitro*. *Brassica oleracea* var. *botrytis* is known to have very low levels of photosynthesis *in vitro* (Grout & Ashton 1978) and this meant that plantlets which were transferred to soil were vulnerable to environmental stress until they had developed a competent photosynthetic system. The transfer from heterotrophic to auxotrophic nutrition does not usually appear to cause any such problem in watercress.

The occurrence of somaclonal variation in regenerated watercress plants is of interest because one of the aims of the work on regeneration from callus was to develop a system which could be used to exploit such somaclonal variation for selection *in vitro* of resistance to crookroot disease of watercress.

5. CONCLUDING DISCUSSION

Watercress is a relatively unimproved crop which must be improved rapidly to meet the demands for high and consistent quality by customers and the need for a disease resistant and high yielding crop by the grower. Tissue culture techniques such as regeneration are attractive as they may be able to provide a more rapid means of plant improvement than traditional breeding methods.

The research into regeneration from various explants of watercress has shown that regeneration from callus is successful and occurs at quite a reasonable frequency through use of the diphenyl-urea-derivative Dropp as the cytokinin. There is scope for increasing still further the regeneration frequency by combining the most effective PGR combinations with the best of the other culture variables tested. The results indicate that the culture variables which should be considered for this further improvement are culture in the light, Gelrite as the gelling agent and possible subculture to PGR-free medium. The various components of casein hydrolysate should be tested to identify the beneficial ones for inclusion in a better defined medium.

Regeneration from gametes i.e. anthers, pollen and ovules was on the whole much more difficult. This was

for several reasons. As discussed earlier very large numbers of anthers have often had to be cultured to detect a very low frequency response to anther culture. This is partly because the biochemical and physiological mechanisms underlying the production of embryos or callus are poorly understood and most of the reported experiments have been empirical. Another problem specific to watercress is the sheer small size of the anthers (around 0.5mm) which means that dissection of anthers is very laborious. This limits the number of anthers that can be tested at any time and it may be that small anthers are more vulnerable to damage during dissection and culture. Culture of isolated pollen was attractive as isolation of the pollen is independent of such problems with anther size and this permitted testing of a wider range of genotypes. However pollen culture is normally seen as more likely to be unsuccessful than anther culture due to the difficulties of keeping isolated pollen viable compared to keeping pollen viable in the closed conditions of the anther. The research was probably limited by failure to find a relatively highly responsive genotype and this was limited by time considerations, limited facilities for raising and initiating-flowers on a wide enough range of genotypes (some genotypes of English watercress had proved recalcitrant in flower formation) and commercial considerations in terms of obtaining a result of direct value to the watercress industry.

The results of ovule culture were initially more promising than those of anther culture in that three possible anther derived structures were formed out of 6,378 anthers cultured whereas 2 calli were formed from the ovules from 48 buds. The results from ovule culture and from anther culture were both limited at the time by lack of a plant regeneration protocol from callus. However now such a regeneration protocol has been developed future workers may feel it is worthwhile carrying out further work on ovule culture, in the hope of being able to regenerate plants from any calli that formed.

Other work has also been carried out to develop a low maintenance system for storing regenerated plants *in vitro* while they can be assessed for disease resistance or improve cropping characteristics. A simple system of storage at 10°C gave considerably improved survival over six months and one year.

Relevant preliminary background work into the breeding system in watercress has been carried out and if, as appears to be the case, watercress consists predominantly of a number of homozygous lines then development of techniques for regeneration from gametes are unnecessary as they are primarily aimed at production of such homozygous lines. It should be possible if a promising plant is regenerated to "bulk it up" *in vitro*, introduce it to the propagation system

and then a crop bed and then expect it to breed true. More work should be carried out to confirm this possibility. Hampshire Watercress Ltd. is using the plants regenerated from callus in this work to look for variation in resistance to crook root disease this winter (1988-1989). However there is unlikely to be any increased resistance as no disease resistance selection pressure was put on the callus. It is hoped that in further work a toxic filtrate of the fungus or *in vitro* infection will be used to impose such selection pressures on the callus and any surviving cells could be regenerated into resistant plants.

In conclusion it can be seen that knowledge of the application of *in vitro* regeneration techniques to watercress has been increased and a protocol for regeneration from callus which is of considerable commercial value has been developed.

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APPENDIX A - MEDIA

Murashige & Skoog (1962)

1.	Macronutrients	for $10m^3$ stock solution use 100ml in $10m^3$ medium
	KNO_3	16.5g
	NH_4NO_3	19.0g
	$MgSO_4.7H_2O$	3.7g
	KH_2PO_4	1.7g
2.	$CaCl_2$	4.4g
		for 100ml stock solution use 5ml in $1dm^3$
3.	$Na_2EDTA.2H_2O$	745mg
	$FeSO_4.7H_2O$	557mg
4.	Micronutrients	use 1ml in $10m^3$
	$MnSO_4.4H_2O$	2,230mg
	$ZnSO_4.4H_2O$	860mg
	H_3BO_3	620mg
	$Na_2MoO_4.2H_2O$	25mg
	$CuSO_4.5H_2O$	2.5mg
	$CoCl_2.6H_2O$	2.5mg

5. Organics & Vitamins use 1ml in $1dm^3$

myo-inositol	10,000mg
glycine	200mg
nicotinic acid	50mg
pyridoxin.HCl	50mg
thiamin.HCl	10mg

6. use 1ml in $1dm^3$

KI	83mg

(store in amber bottle at 4°C)

Gamborg's B^5 medium (1968)

1. Macronutrients for $1dm^3$ stock solution
use 100ml in $1dm^3$ medium

KNO_3	25g
$NaH_2PO_4.H_2O$	1.5g
$(NH_4)_2SO_4$	1.34g
$MgSO_4.7H_2O$	2.5g

2. for 100ml stock solution use
1ml in $1dm^3$ medium

$CaCl_2.2H_2O$	15g

3. use 5ml in 1dm^3

$Na_2EDTA.2H_2O$	745mg
$FeSO_4.7H_2O$	557mg

4. Micronutrients use 1ml in 1dm^3

$MnSO_4.H_2O$	1,000mg
H_3BO_3	300mg
$ZnSO_4.7H_2O$	200mg
$Na_2MoO_4.2H_2O$	25mg
$CuSO_4.SH_2O$	2.5mg

5. Organics & Vitamins use 1ml in 1dm^3

nicotinic acid	100mg
thiamin.HCl	1,000mg
pyridoxin.HCl	100mg
myo-inositol	10,000mg

6. use 1ml in 1dm^3

KI	75mg

(store in amber bottle at 4°C)

Nitsch & Nitsch (1969) medium

1.	Macronutrients	for 1dm³ stock solution use 100ml in 1dm³ medium
	KNO_3	9.5g
	NH_4NO_3	1.5g
	$MgSO_4.7H_2O$	1.855g
	KH_2PO_4	0.68g
2.		for 100ml stock solution use 1ml in 1dm³ medium
	$CaCl_2$	15g
3.	Micronutrients	for 100ml stock solution use 1ml in 1dm³
	$MnSO_4.4H_2O$	2,500mg
	H_3BO_3	1,000mg
	$ZnSO_4.7H_2O$	1,000mg
	$Na_2MoO_4.2H_2O$	250mg
	$CuSO_4.5H_2O$	25mg
4.		use 5ml in 1dm³ medium
	Na_2EDTA	745mg
	$FeSO_4.7H_2O$	557mg

5. Organics & Vitamins use 1ml in $1dm^3$

glycine	200mg
nicotinic acid	500mg
pyridoxin.HCl	50mg
thiamin.HCl	50mg
folic acid	50mg
biotin	5mg

APPENDIX B

TABLE 1. Exp. 1. Effect of cytokinin and auxin on shoot regeneration

	CYTOKININ		AUXIN		REPLICATIONS							ROW
EX	DROPP	BA	NAA	24D	R1	R2	R3	R4	R5	R6	R7	TTL
1	1	0	1	0	0	0	0	0	0	0	0	0
1	1	0	2	0	0	0	0	0	0	0	0	0
1	1	0	3	0	0	0	0	0	0	0	0	0
1	1	0	4	0	0	0	0	0	0	0	0	0
2	1	0	0	1	0	0	0	0	0	0	0	0
2	1	0	0	2	0	0	0	0	0	0	0	0
2	1	0	0	3	0	0	0	0	0	0	0	0
2	1	0	0	4	0	0	0	0	0	0	0	0
3	0	1	1	0	0	0	0	0	0	0	0	0
3	0	1	2	0	0	0	0	0	0	0	0	0
3	0	1	3	0	0	0	0	0	0	0	0	0
3	0	1	4	0	0	0	0	0	0	0	0	0
4	0	1	0	1	0	0	0	0	0	0	0	0
4	0	1	0	2	0	0	0	0	0	0	0	0
4	0	1	0	3	0	0	0	0	0	0	0	0
4	0	1	0	4	0	0	0	0	0	0	0	0
1	2	0	1	0	0	0	0	0	0	0	0	0
1	2	0	2	0	1	0	1	0	0	2	0	4
1	2	0	3	0	1	1	0	1	0	1	0	4
1	2	0	4	0	0	0	0	1	0	0	2	3
2	2	0	0	1	0	0	1	0	0	0	0	1
2	2	0	0	2	0	1	1	0	0	1	0	3
2	2	0	0	3	0	1	0	3	0	0	0	4
2	2	0	0	4	0	1	0	0	0	0	0	1
3	0	2	1	0	0	0	0	0	0	0	0	0
3	0	2	2	0	0	0	0	1	0	0	0	1
3	0	2	3	0	0	0	0	0	0	0	0	0
3	0	2	4	0	0	0	0	0	0	0	0	0
4	0	2	0	1	0	0	0	0	0	0	0	0
4	0	2	0	2	0	0	0	0	0	0	0	0
4	0	2	0	3	0	0	0	0	0	0	0	0
4	0	2	0	4	0	0	0	0	0	0	0	0
1	3	0	1	0	0	0	0	0	0	0	0	0
1	3	0	2	0	0	0	0	0	0	0	0	0
1	3	0	3	0	0	0	1	1	0	0	0	2
1	3	0	4	0	0	0	0	0	0	0	0	0
2	3	0	0	1	0	0	0	0	0	0	0	0
2	3	0	0	2	0	5	8	4	0	0	0	17
2	3	0	0	3	1	7	1	0	0	0	9	18
2	3	0	0	4	0	0	0	0	0	0	0	0
3	0	3	1	0	0	0	0	0	0	0	0	0
3	0	3	2	0	0	0	0	0	0	0	0	0
3	0	3	3	0	0	0	2	1	0	0	2	5
3	0	3	4	0	0	1	0	0	0	4	0	5
4	0	3	0	1	0	0	0	1	0	2	0	3
4	0	3	0	2	0	0	0	0	0	0	0	0
4	0	3	0	3	0	0	0	0	0	0	0	0
4	0	3	0	4	0	0	0	1	0	1	0	2
1	4	0	1	0	0	1	0	0	0	0	0	1
1	4	0	2	0	0	0	0	0	0	1	0	1
1	4	0	3	0	0	2	0	0	1	1	1	5
1	4	0	4	0	0	0	0	1	1	1	1	4
2	4	0	0	1	0	0	0	0	0	0	0	0
2	4	0	0	2	11	0	7	0	11	9	0	38

TABLE 1 cont'd.

	CYTOKININ		AUXIN		REPLICATIONS							ROW
EX	DROPP	BA	NAA	24D	R1	R2	R3	R4	R5	R6	R7	TTL
2	4	0	0	3	0	0	1	1	0	1	1	4
2	4	0	0	4	1	1	0	2	0	0	0	4
3	0	4	1	0	0	0	0	0	0	0	0	0
3	0	4	2	0	0	0	0	0	0	0	0	0
3	0	4	3	0	0	0	1	0	0	0	0	1
3	0	4	4	0	0	0	3	0	0	0	0	3
4	0	4	0	1	0	0	0	0	0	0	0	0
4	0	4	0	2	0	2	0	1	1	0	0	4
4	0	4	0	3	0	0	1	0	0	0	2	3
4	0	4	0	4	0	0	0	1	0	0	0	1
					15	23	28	20	14	24	18	142

Exp. 1.

ANOVAR TABLES cf= 45.0

ITEMS	SOURCE	SS	DF	MS	F	SIG	99% F
7	REPS	2.3973	6	0.3996	0.3744	NS	2.6
64	TRTS	284.1339	63	4.5101	4.2261	***	1.5
	RESID	388.4598	364	1.0672			
	missing	data	14				
448	TOTAL	674.9911	447				

ITEMS	SOURCE	SS	DF	MS	F	SIG	99% F
7	REPS	2.3973	6	0.3996	0.3744	NS	2.6
(64	TRTS)						
8	CYTOK'S	51.2054	7	7.3151	6.8545	***	2.6
8	AUXINS	49.9196	7	7.1314	6.6823	***	2.6
	CK.x.AX	183.0089	49	3.7349	3.4997	***	1.5
	RESIDUAL	388.4598	364	1.0672			
	missing	data	14				
448	TOTAL	674.9911	447				

F at 63 on 364 d.f. at P=0.05 approx. 1.3
Scheffe's critical difference value= 4.99

TABLE 2. Exp. 2. Influence of culture variables on shoot regeneration.

trt name	Dropp level	No. of shoots produced Replication number 1	2	3	4	5	6	7	TTL
1 Gelrite	1	2	0	0	0	2	0	8	12
	2	1	0	4	0	0	0	0	5
	3	0	0	0	0	2	0	0	2
2 MC29 agar	1	1	2	0	0	1	0	0	4
	2	1	0	0	1	0	0	0	2
	3	5	0	0	0	0	2	0	7
3 MC2 agar	1	0	0	0	4	1	0	0	5
	2	0	0	0	2	0	0	0	2
	3	0	0	0	0	3	0	1	4
4 CHa250mg/l	1	0	1	0	0	1	0	0	2
	2	5	0	2	0	0	1	0	8
	3	0	0	2	0	0	0	14	16
5 CHa500mg/l	1	0	1	0	0	0	0	0	1
	2	0	0	0	2	1	1	0	4
	3	1	0	1	0	1	0	0	3
6 Dark	1	0	0	0	0	0	0	0	0
	2	0	0	0	1	0	0	0	1
	3	0	1	2	1	0	0	1	5
7 Light	1	1	2	6	0	0	4	0	13
	2	2	1	0	4	4	12	0	23
	3	7	1	1	1	2	0	3	15
8 +NAA	1	0	0	1	0	1	0	0	2
	2	0	2	0	0	0	0	0	2
	3	0	0	0	0	0	1	0	1
9 Hormone	1	0	0	0	1	0	0	0	1
free	2	0	1	0	4	4	0	1	10
	3	2	4	0	0	0	2	0	8
10 Leaves	1	0	0	0	0	0	0	0	0
explanted	2	1	1	0	0	3	0	1	6
	3	0	0	0	0	0	0	1	1
TOTALS		29	17	19	21	26	23	30	165

TABLE 3. Exp. 2. Influence of culture variables on explant response.

		No. of productive explants							
trt	Dropp	Replication number							
name	level	1	2	3	4	5	6	7	ttl
1 Gelrite	1	1	0	0	0	2	0	2	5
	2	1	0	2	0	0	0	0	3
	3	0	0	0	0	1	0	0	1
2 MC29 agar	1	1	2	0	0	1	0	0	4
	2	1	0	0	1	0	0	0	2
	3	2	0	0	0	0	1	0	3
3 MC2 agar	1	0	0	0	2	1	0	0	3
	2	0	0	0	1	0	0	0	1
	3	0	0	0	0	2	0	1	3
4 CH@250mg/l	1	0	1	0	0	1	0	0	2
	2	2	0	1	0	0	1	0	4
	3	0	0	1	0	0	0	3	4
5 CH@500mg/l	1	0	1	0	0	0	0	0	1
	2	0	0	0	1	1	1	0	3
	3	1	0	1	0	1	0	0	3
6 Dark	1	0	0	0	0	0	0	0	0
	2	0	0	0	1	0	0	0	1
	3	0	1	1	1	0	0	1	4
7 Light	1	1	1	3	0	0	2	0	7
	2	1	1	2	2	0	5	0	11
	3	1	1	1	1	2	0	1	7
8 +NAA	1	0	0	1	0	1	0	0	2
	2	0	1	0	0	0	0	0	1
	3	0	0	0	0	0	1	0	1
9 Hormone	1	0	0	0	1	0	0	0	1
free	2	0	1	0	2	1	0	1	5
	3	1	2	0	0	0	1	0	4
10 Leaves	1	0	0	0	0	0	0	0	0
explanted	2	1	1	0	0	1	0	1	4
	3	0	0	0	0	0	0	1	1
TOTALS		14	13	13	13	15	13	11	91

APPENDIX C - Data for effect of clonal seed parents.

TABLE 1. plant heights (cm)

C1-C4 = control heights
C4-C8 = x heights
C9-C12 = y heights

ROW	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
1	85	97	90	126	88	135	113	97	134	115	136	96
2	87	102	97	130	90	135	117	100	142	137	139	143
3	95	106	98	130	90	137	135	108	145	174	139	144
4	97	112	105	133	92	137	136	111	147	175	150	147
5	97	120	110	136	96	140	140	114	150	175	153	148
6	103	120	110	155	99	141	147	115	153	178	157	148
7	106	126	115	162	104	141	149	118	154	179	157	155
8	114	134	118	163	104	142	149	120	160	183	159	157
9	116	143	118	172	105	144	150	129	161	184	161	157
10	120		120		110	144	152	133	162	184	161	157
11	124		120		112	145	153		166	186	161	160
12	126		122		117	145	154		176	187	164	160
13	128		124		118	147	159		180	194	167	161
14	131		126		124	148	160		184	206	172	183
15	132		128		128	153	166		199	207	175	163
16	136		129			157	167		204	208	175	166
17	136					180	179			211	175	170
18	142									218	176	171
19	148									220	177	172
20	153									220	179	174
21	159									222	181	176
22											205	177
23												178
24												179
25												182
26												184
27												187
28												209

TABLE 2. statistical description of plant heights.

	N	MEAN	MEDIAN	TRMEAN	STDEV	SEMEAN	MIN	MAX	Q1	Q3
C1	21	120.71	124.00	120.58	21.56	4.70	85.00	159.00	100.00	136.00
C2	9	117.78	120.00	117.78	15.12	5.04	97.00	143.00	104.00	130.00
C3	16	114.37	118.00	115.07	11.74	2.93	90.00	129.00	106.25	123.50
C4	9	145.22	136.00	145.22	17.60	5.87	126.00	172.00	130.00	162.50
C5	15	105.13	104.00	104.69	12.83	3.31	88.00	128.00	92.00	117.00
C6	17	145.35	144.00	143.73	10.72	2.60	135.00	180.00	138.50	147.50
C7	17	148.59	150.00	148.93	16.87	4.09	113.00	179.00	138.00	159.50
C8	10	114.50	114.50	114.37	11.38	3.60	97.00	133.00	106.00	122.25
C9	16	163.56	160.50	162.79	20.18	5.04	134.00	204.00	147.75	179.00
C10	21	188.71	186.00	190.84	26.88	5.87	115.00	222.00	176.50	209.50
C11	22	164.50	162.50	163.90	16.09	3.43	136.00	205.00	156.00	175.25
C12	28	163.71	163.00	164.58	20.00	3.78	96.00	209.00	155.50	176.75

TABLE 3. 2nd internode lengths (mm)

C13-16 = control internodes
C17-20 = x internodes
C21-24 = y internodes

ROW	C13	C14	C15	C16	C17	C18	C19	C20	C21	C22	C23	C24
1	7	8	7	13	4	15	9	4	11	18	13	13
2	8	11	8	14	5	16	10	8	12	20	18	15
3	8	13	8	15	8	17	13	9	14	22	20	17
4	9	16	12	18	8	17	14	11	15	24	21	18
5	13	17	12	20	11	18	17	13	17	25	21	19
6	14	18	12	20	13	18	17	15	19	25	22	20
7	14	19	13	25	13	19	18	15	20	30	23	20
8	15	20	13	28	13	19	18	15	20	30	24	20
9	16	26	13	34	14	21	19	16	22	30	24	23
10	17		15		14	22	19	28	25	32	24	24
11	17		15		17	23	21		25	33	25	24
12	17		16		17	26	21		27	34	26	24
13	18		17		20	26	22		28	35	29	24
14	18		20		21	28	27		29	37	30	24
15	20		25		26	28	28		34	38	32	25
16	20		32			28	30		35	40	32	25
17	20					29	30			40	33	26
18	22									40	33	27
19	25									40	33	28
20	28									42	38	29
21	30									44	38	31
22											43	31
23												33
24												34
25												34
26												38
27												39
28												40

TABLE 4. Statistical analysis of internode lengths.

	N	MEAN	MEDIAN	TRMEAN	STDEV	SEMEAN	MIN	MAX	Q1	Q3
C13	21	16.95	17.00	16.79	6.22	1.36	7.00	30.00	13.50	20.00
C14	9	16.44	17.00	16.44	5.32	1.77	8.00	26.00	12.00	19.50
C15	16	14.87	13.00	14.21	6.43	1.61	7.00	32.00	12.00	16.75
C16	9	20.78	20.00	20.78	7.01	2.34	13.00	34.00	14.50	26.50
C17	15	13.60	13.00	13.38	6.03	1.56	4.00	26.00	8.00	17.00
C18	17	21.76	21.00	21.73	4.84	1.17	15.00	29.00	17.50	27.00
C19	17	19.59	19.00	19.60	6.37	1.55	9.00	30.00	15.50	24.50
C20	10	13.40	14.00	12.75	6.42	2.03	4.00	28.00	8.75	15.25
C21	16	22.06	21.00	21.93	7.38	1.84	11.00	35.00	15.50	27.75
C22	21	32.33	33.00	32.47	7.70	1.68	18.00	44.00	25.00	40.00
C23	22	27.36	25.50	27.30	7.36	1.57	13.00	43.00	21.75	33.00
C24	28	25.89	24.50	25.85	7.13	1.35	13.00	40.00	20.00	31.00

TABLE 5. 3rd internode lengths (mm)

C25-28 = control internodes
C29-32 = x internodes
C33-36 = y internodes

ROW	C25	C26	C27	C28	C29	C30	C31	C32	C33	C34	C35	C36
1	7	8	9	6	7	9	12	6	13	22	13	15
2	9	10	10	13	8	12	13	7	16	22	18	16
3	10	15	10	13	8	15	16	9	17	22	20	18
4	10	16	10	21	8	16	17	10	19	24	20	19
5	11	17	10	25	10	17	17	11	19	25	20	19
6	12	20	11	25	10	17	18	12	20	26	21	19
7	14	20	13	26	13	20	20	14	20	27	21	20
8	14	20	16	30	14	21	22	16	22	27	22	20
9	14	21	16	36	17	21	22	16	22	27	22	21
10	15		18		17	23	23	16	22	27	22	22
11	15		18		18	24	24		22	28	23	22
12	16		18		18	25	25		23	30	23	23
13	16		19		19	26	28		23	32	24	23
14	18		19		25	28	30		23	33	24	26
15	18		21		25	29	31		24	34	25	27
16	19		22			29	33		24	36	28	27
17	20					31	35			37	30	28
18	22									38	30	29
19	22									38	30	30
20	22									42	31	30
21	22									46	32	31
22											33	32
23												32
24												32
25												33
26												34
27												38
28												39

TABLE 6. Statistical analysis of internode lengths.

3rd internodes

	N	MEAN	MEDIAN	TRMEAN	STDEV	SEMEAN	MIN	MAX	Q1	Q3
C25	21	15.52	15.00	15.63	4.63	1.01	7.00	22.00	11.50	19.50
C26	9	16.33	17.00	16.33	4.66	1.55	8.00	21.00	12.50	20.00
C27	16	15.00	16.00	14.93	4.49	1.12	9.00	22.00	10.00	18.75
C28	9	21.67	25.00	21.67	9.43	3.14	6.00	36.00	13.00	28.00
C29	15	14.47	14.00	14.23	6.00	1.55	7.00	25.00	8.00	18.00
C30	17	21.35	21.00	21.53	6.36	1.54	9.00	31.00	16.50	27.00
C31	17	22.71	22.00	22.60	6.91	1.68	12.00	35.00	17.00	29.00
C32	10	11.70	11.50	11.88	3.74	1.18	6.00	16.00	8.50	16.00
C33	16	20.563	22.000	20.857	3.119	0.780	13.000	24.000	19.000	23.000
C34	21	30.62	28.00	30.26	6.87	1.50	22.00	46.00	25.50	36.50
C35	22	24.18	23.00	24.30	5.14	1.10	13.00	33.00	20.75	30.00
C36	28	25.89	26.50	25.81	6.62	1.25	15.00	39.00	20.00	31.75

TABLE 7. Leaf ratios (leaf length:leaf width)

C46-49 + C58-61 = control leaf ratios
C50-53 + C62-65 = x leaf ratios
C54-57 + C66-69 = y leaf ratios

	leaf ratios											
ROW	C46	C47	C48	C49	C50	C51	C52	C53	C54	C55	C56	C57
1	1.040	1.150	0.938	1.000	1.143	0.917	0.950	0.882	1.000	1.052	1.056	1.000
2	0.962	0.900	0.963	1.000	1.111	0.889	0.929	0.882	0.957	1.056	1.143	1.000
3	0.963	1.083	1.000	1.067	1.125	1.000	1.059	1.133	1.091	0.917	1.000	1.000
4	0.867	1.000	0.938	0.895	0.882	0.913	0.808	0.950	1.045	0.864	0.850	1.000
5	0.950	0.950	0.952	1.000	1.056	0.947	1.063	1.048	1.095	1.042	1.052	1.111
6	1.000	1.056	0.947	1.000	1.000	0.944	1.095	1.000	0.870	1.052	1.167	1.133
7	1.000	0.938	1.053	1.176	0.818	0.800	0.875	0.813	0.947	1.111	0.909	0.956
8	1.059	1.118	1.000	0.944	1.000	1.056	0.917	0.857	1.052	1.056	1.063	1.118
9	0.938	1.000	1.000	1.000	1.000	0.941	0.870	0.840	0.944	0.895	0.955	1.083
10	0.917		1.095		1.000	1.000	1.000	0.833	0.900	0.950	1.118	0.950
11	0.880		0.952		0.933	1.071	0.889		1.000	1.111	1.000	1.125
12	0.955		0.938		0.867	0.833	0.833		1.105	1.133	1.294	1.200
13	1.067		1.167		0.800	1.176	1.188		0.850	0.944	1.056	1.133
14	1.150		1.053		0.944	1.000	1.000		1.043	1.059	1.056	1.176
15	1.111		1.133		0.842	1.143	1.231		1.000	1.063	0.813	1.063
16	1.000		0.857			1.000	1.000		1.091	1.125	0.905	1.059
17	1.143					1.000	0.889			1.150	1.071	1.118
18	0.900									1.200	1.125	1.063
19	0.926									1.105	1.000	1.063
20	1.067									1.063	1.050	0.895
21	0.778									1.200	1.058	1.150
22											0.947	1.000
23												1.154
24												0.938
25												1.000
26												1.188
27												1.125
28												1.000

ROW	C58	C59	C60	C61	C62	C63	C64	C65	C66	C67	C68	C69
1	1.034	1.042	1.000	0.947	1.176	0.956	0.880	0.909	0.935	1.158	1.042	1.056
2	1.000	0.952	1.033	1.050	1.043	0.905	0.875	0.947	1.160	1.095	1.185	1.059
3	1.000	1.080	0.864	0.947	1.063	1.000	1.095	1.100	1.063	0.885	0.947	1.074
4	0.955	1.105	0.941	0.833	0.957	0.893	0.914	0.909	1.074	0.846	1.000	1.222
5	0.857	1.000	0.905	1.038	1.000	0.909	1.000	1.167	1.000	1.040	1.000	1.048
6	1.000	1.000	1.000	1.000	1.100	1.111	0.960	1.000	1.040	1.150	1.182	1.059
7	0.913	1.000	1.130	1.000	0.964	0.850	0.880	0.850	1.105	1.056	0.963	0.920
8	0.875	1.143	1.045	0.913	1.056	1.059	0.857	0.857	0.963	1.050	0.950	1.100
9	1.000	1.000	1.059	1.050	1.000	0.938	0.875	0.920	1.045	0.954	1.083	1.077
10	0.944		1.045		0.875	1.100	1.000	0.913	0.857	0.917	1.053	0.864
11	0.967		1.043		0.889	0.933	0.944		1.053	1.167	1.045	1.050
12	1.037		1.000		0.850	0.846	0.800		1.174	1.167	1.231	1.000
13	0.952		1.186		0.964	1.100	1.100		0.885	0.947	1.211	1.105
	1.167											
14	1.053		1.059		1.000	1.056	1.000		1.077	1.100	1.100	1.095
15	0.909		0.933		0.920	1.071	1.067		1.000	1.056	0.850	1.053
16	1.071		0.938			1.067	0.870		1.160	1.050	0.958	1.111
17	1.000					0.938	0.870			1.043	1.000	1.048
18	1.000									1.136	1.063	1.000
19	1.067									1.143	1.135	1.118
20	0.923									1.200	1.045	0.870
21										1.105	1.091	1.143
22											1.000	0.870
23												1.059
24												0.944
25												1.053
26												1.000
27												1.000
28												1.167

TABLE 8. Statistical description of leaf ratios.

	N	MEAN	MEDIAN	TRMEAN	STDEV	SEMEAN	MIN	MAX	Q1	Q3
C46	21	0.9844	0.9630	0.9866	0.0946	0.0207	0.7780	1.1500	0.9215	1.0630
C47	9	1.0217	1.0000	1.0217	0.0856	0.0285	0.9000	1.1500	0.9440	1.1005
C48	16	0.9991	0.9815	0.9973	0.0820	0.0205	0.8570	1.1670	0.9403	1.0530
C49	9	1.0091	1.0000	1.0091	0.0782	0.0261	0.8950	1.1760	0.9720	1.0335
C50	15	0.9681	1.0000	0.9675	0.1113	0.0287	0.8000	1.1430	0.8670	1.0560
C51	17	0.9782	1.0000	0.9769	0.0986	0.0239	0.8000	1.1760	0.9150	1.0280
C52	17	0.9762	0.9500	0.9705	0.1204	0.0292	0.8080	1.2310	0.8820	1.0610
C53	10	0.9238	0.8820	0.9115	0.1060	0.0335	0.8130	1.1330	0.8383	1.0120
C54	16	0.9994	1.0000	1.0025	0.0818	0.0205	0.8500	1.1050	0.9448	1.0813
C55	21	1.0547	1.0590	1.0571	0.0937	0.0204	0.8640	1.2000	0.9960	1.1180
C56	22	1.0313	1.0540	1.0291	0.1090	0.0232	0.8130	1.2940	0.9530	1.0828
C57	28	1.0643	1.0630	1.0656	0.0828	0.0156	0.8950	1.2000	1.0000	1.1310
C58	21	0.9869	1.0000	0.9842	0.0726	0.0159	0.8570	1.1670	0.9335	1.0355
C59	9	1.0358	1.0000	1.0358	0.0617	0.0206	0.9520	1.1430	1.0000	1.0925
C60	16	1.0113	1.0165	1.0094	0.0828	0.0207	0.8640	1.1860	0.9388	1.0555
C61	9	0.9753	1.0000	0.9753	0.0725	0.0242	0.8330	1.0500	0.9300	1.0440
C62	15	0.9905	1.0000	0.9870	0.0886	0.0229	0.8500	1.1760	0.9200	1.0560
C63	17	0.9842	0.9560	0.9850	0.0913	0.0221	0.8460	1.1110	0.9070	1.0690
C64	17	0.9404	0.9140	0.9391	0.0900	0.0218	0.8000	1.1000	0.8725	1.0000
C65	10	0.9572	0.9165	0.9444	0.1032	0.0326	0.8500	1.1670	0.8960	1.0250
C66	16	1.0369	1.0490	1.0400	0.0937	0.0234	0.8570	1.1740	0.9723	1.0980
C67	21	1.0602	1.0560	1.0642	0.0999	0.0218	0.8460	1.2000	0.9970	1.1465
C68	22	1.0515	1.0450	1.0526	0.0960	0.0205	0.8500	1.2310	0.9908	1.1087
C69	28	1.0416	1.0545	1.0415	0.0871	0.0165	0.8640	1.2220	1.0000	1.0987

TABLE 9. Nos. of marketable, unmarketable and total stems.

conmark = control marketable stems
conunmark = control unmarketable stems
contotal = control total stems
xmark = x marketable stems
xunmark = x unmarketable stems
xtotal = x total stems
ymark = y marketable stems
yunmark = y- unmarketable stems
ytotal = y total stems

ROW	conmark	conunmark	contotal	xmark	xunmark	xtotal	ymark	yunmark	ytotal
1	21	18	39	16	27	43	15	21	36
2	34	32	66	16	10	26	16	14	30
3	13	10	23	22	31	53	26	21	47
4	17	10	27	14	10	24	17	15	32
5	11	3	14	23	18	41	19	8	27
6	9	8	17	18	7	25	17	11	28
7	17	14	31	17	31	48	21	23	44
8	11	12	23	19	25	44	28	43	68
9	14	17	31	11	14	25	20	26	46
10	16	37	53	22	24	46	22	26	48
11	16	13	29	23	25	48	17	12	29
12	15	26	41	17	33	50	22	31	53
13	16	12	28	11	11	22	25	27	52
14	15	18	33	15	15	30	15	11	26
15	9	13	22	11	15	26	28	21	49
16	19	20	39	10	12	22	16	24	40